The Association of Bone and Cartilage in Matrix Proteolysis of Articular Cartilage, and its Role in Palmar/Plantar Osteochondral Disease in the Thoroughbred Racehorse

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To my parents, Moira and John,

for their continued support, love and encouragement.

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Introduction and Review of the Literature

1.1 Overview

Synovial joints are composed of different tissues (cartilage, calcified cartilage, subchondral bone, synovium, and intra- and extra-articular ligaments). In the normal joint, these tissues interact to maintain function and physiological balance. In disease, when one tissue deteriorates, there is an effect on the other tissues such that osteoarthritis may be considered a form of "organ disease".

Much of the research emphasis in osteoarthritis (OA) has been placed on the biological and mechanical mechanisms of cartilage breakdown. Subchondral bone involvement has traditionally been viewed as a secondary event, resulting from progression of the pathology of the articular cartilage. However, this classical view of OA has been questioned and the time frame of subchondral bone injury in OA remains a point of debate. The questions remain whether the articular cartilage is affected first, then the subchondral bone, or vice versa. Furthermore, the mechanisms by which subchondral bone interact with articular cartilage and how this occurs *in vivo* are at present unclear.

1.2 Anatomy of the Synovial Joint

The articular cartilage provides the primary weight-bearing surface in the synovial joint. The cartilage is composed of a collagen (predominantly type-II, but also types- VI, IX, X and XI) and proteoglycan matrix, with chondrocytes as the cellular component and up to 80% water (Buckwalter and Mankin 1998). The tidemark forms the junction between the articular cartilage and the underlying calcified cartilage layer. Deep to the calcified cartilage lies the subchondral bone plate, which is cortical bone (Burr 2004). The subchondral bone plate is supported from beneath by the trabecular subchondral bone. Therefore, the joint contains three mineralised tissues (calcified cartilage, subchondral bone plate and subchondral trabecular bone), which differ mechanically and physiologically, and respond to drugs and mechanical forces in different ways (Burr 2004).

It has been shown that although the hyaline articular cartilage is avascular and there is no direct vascular connection between subchondral bone and articular cartilage, there is not an absolute barrier to diffusion of nutrients between the two structures (Imhof et al. 1999). Furthermore, the presence of clefts running across the subchondral bone into the calcified cartilage and deep layers of articular cartilage, have been described in the literature under a number of synonymous names including vascular invasion (Harrison *et al.* 1953), vascular channels (Clark 1990), microcracks (Mori *et al.* 1993; Sokoloff 1993) and subchondral bone resorption pits (Chambers *et al.* 1984). Subchondral bone resorption pits have been implicated in cartilage nutrition and cross-talk between subchondral bone and cartilage (Lajeunesse and Reboul 2003) and recently have been shown to express MMPs and degrade proteoglycan from the articular cartilage around the tip of the invasion (Shibakawa *et al.* 2005).

1.3 Pathophysiology of Osteoarthritis

OA is characterised by degeneration of articular cartilage, intra-articular inflammation with synovitis, and changes in peri-articular and subchondral bone (Goldring and Goldring 2007). The pathogenesis of OA is multifactorial including ageing, mechanical factors, and genetic factors, and may be considered to result from abnormal loading of a normal joint, or normal loading of an abnormal joint (Figure 1.1)

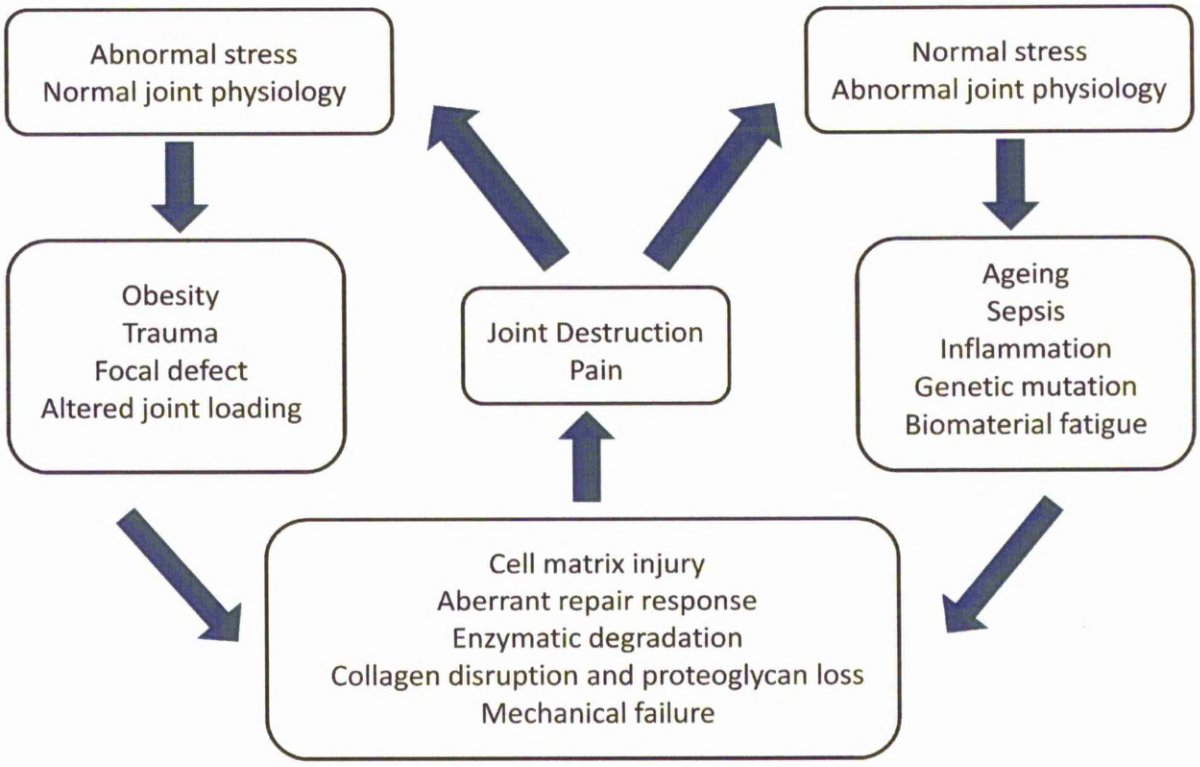

Figure 1.1: Mechanisms involved in the pathogenesis of osteoarthritis

OA can be considered to be a progressive disease, commencing with initiation, continuing through early stage disease and culminating in late-stage, irreversible pathological changes in hyaline articular cartilage. Various inflammatory cytokines are involved in the disease process and the cellular and morphological changes in hyaline articular cartilage are as outlined in Figure 1.2 (Goldring and Goldring 2007).

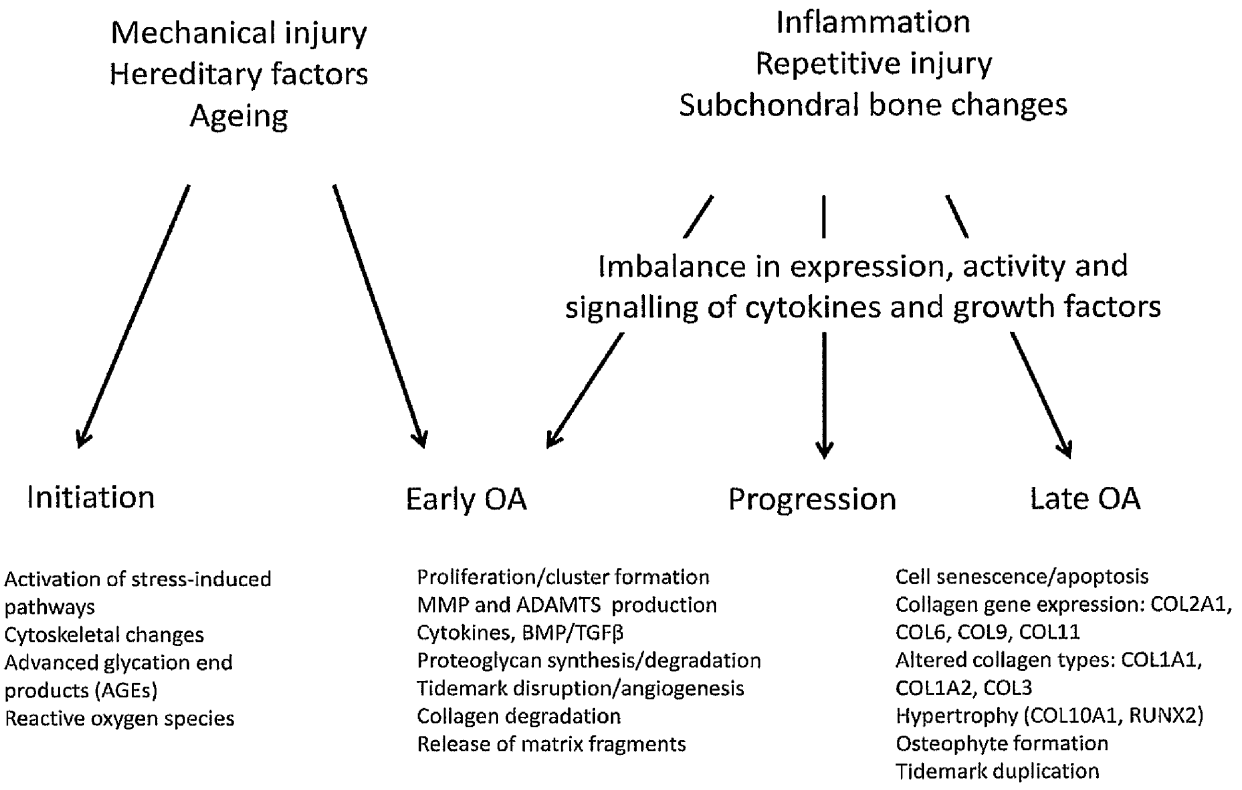

Figure 1.2: Events involved in the initiation of OA and progression to late stage OA. Potential causative factors and cellular and morphological alterations are listed.

In the early stage of disease there may be an upregulation of synthetic activity mediated via a chondrocyte response. In the human the earliest changes in the cartilage of an unstable knee joint have been reported to be enhanced synthesis of matrix with an increased content of aggrecan (McDevitt *et al.* 1977). This is followed by a phase of increased matrix turnover with net depletion of principal matrix components. Finally, in the severe and end stages of disease, damage to and loss of the collagen network is observed (Brandt *et al.* 1991; Guilak *et al.* 1994; Nelson *et al.* 2006; Setton *et al.* 1994).

Metalloproteinases and aggrecanases are generally considered to play a principal role in the cleavage of matrix macromolecules, including type II collagen and the cartilage proteoglycan aggrecan. Collagenases (MMP -1, -8, -13 and -14) cleave type II collagen at a specific site, resulting in denaturation of the α chains, which are then susceptible to secondary cleavage by collagenases and

other MMPs such as MMP-3, -2 and -9. Aggrecan is cleaved by various MMPs and also ADAMTS -4 and -5 (Moskowitz *et al.* 2007).

Collagenase activity, the denaturation of type II collagen and its associated loss are much increased in osteoarthritic cartilage (Billinghurst *et al.* 1997; Hollander *et al.* 1995; Squires *et al.* 2003). In established, or end-stage OA, the synthesis of type II collagen is simultaneously markedly increased (Aigner *et al.* 1993; Aurich *et al.* 2005; Matyas *et al.* 1997; Nelson *et al.* 1998). However, the newly synthesised molecules, as well as the pre-existing collagen are subjected to excessive proteolysis (Dahlberg *et al.* 2000). Aggrecan synthesis is also increased in OA, but is concurrently subjected to excessive proteolysis (Rizkalla *et al.* 1992).

Proteolysis can be potentiated as a result of degradation products of matrix molecules stimulating degradation through chondrocyte and synovial cell-mediated activation, thus setting up a chronic cycle (Moskowitz *et al.* 2007). Fibronectin is produced in increased amounts in OA cartilage (Chevalier *et al.* 1996) and it has been shown that different fragments of fibronectin can stimulate chondrocyte-mediated cartilage resorption by cell surface receptor activation (Homandberg *et al.* 1992; Yasuda and Poole 2002). Similarly, fragments of type II collagen have also been shown to be capable of inducing matrix resorption (Yasuda *et al.* 2006).

1.4 Models of Osteoarthritis

The understanding of the pathogenesis of OA lies not only in investigation of late stage disease, but also investigation of the early stages of disease. In human studies, *ex vivo* tissue is obtained predominantly from arthroplasty (e.g. knee replacement) patients whereby there are already pre-existing severe changes in the hyaline articular cartilage and subchondral bone. As tissue from early stage disease is rarely available for research purposes, there is a large degree of reliance on animal models.

Over the last 2 years, studies on at least 25 different models of OA have been reported (Ameye and Young 2006). Each has their own advantages, disadvantages, proponents and opponents, suggesting that no one model is completely ideal for investigation of OA. Various models have been used to describe simultaneous osteoarthritic changes in cartilage and bone of various experimental species (Table 1.1). Techniques commonly utilised include surgically induced instability models such as partial menisectomy (Moskowitz et al. 1973) and anterior cruciate ligament transaction (Pond and Nuki 1973) and models using animals known to be affected by naturally occurring spontaneous OA e.g. the Hartley guinea pig (Bendele and Hulman 1988).

More recently, in an attempt to characterise the differences between bone and cartilage induced OA, further models of OA have been developed. The canine groove model (Mastbergen et al. 2006) whereby OA is induced by the surgical creation of defects in the articular cartilage without damage to the underlying subchondral bone or creation of joint instability, is an example of a primarily cartilage-driven OA. Another canine model (Lahm et al. 2005; Lahm et al. 2004; Mrosek et al. 2006) may also be used to investigate subchondral bone driven OA. In this model, impact is applied to the subchondral bone without surgical invasion of the joint and without damage to the articular cartilage. After impact, there is immediate evidence of subchondral bleeding, oedema and microfractures, with cartilage degeneration developing over subsequent months.

Type of Model	Species	References
Surgically Induced		
Anterior cruciate ligament resection	Mouse, rat, rabbit, dog ,cat	(Boyd *et al.* 2005; Clements *et al.* 2003; DeGroot *et al.* 2004; Hayami *et al.* 2006; Inoue *et al.* 2006; Kamekura *et al.* 2005)
Menisectomy	Mouse, sheep, guinea pig, rat	(Cake *et al.* 2004; Kamekura *et al.* 2005; Moore *et al.* 2005; Quasnichka *et al.* 2005)
Ovariectomy	Rat, sheep	(Cake *et al.* 2005; Hoegh-Andersen *et al.* 2004)
Articular groove	Dog	(Mastbergen *et al.* 2006)
Partial and full cartilaginous thickness defect	Rabbit, rat	(Kuroda *et al.* 2006; Yamamoto *et al.* 2004)
Transarticular impact to induce trauma	Dog	(Lahm *et al.* 2004)
Carpal chip fracture	Horse	(Frisbie *et al.* 2002)
Collateral metacarpophalangeal and lateral collateral sesamoidean ligament resection	Horse	(Simmons *et al.* 1999)
Enzymatically/Chemically Induced		
Collagenase induced	Mouse	(Blom *et al.* 2004)
TGFβ	Mouse	(van Lent *et al.* 2004)
Iodoacetate injection	Mouse, horse	(Bove *et al.* 2003; Fernihough *et al.* 2004; Gustafson *et al.* 1992)
Lipolysaccharide injection	Mouse, horse	(Todhunter *et al.* 1996; Tsuchiya *et al.* 2005)
Spontaneous		
STR/ort	Mouse	(Regan *et al.* 2005)
Natural ageing	Rat, mouse, guinea pig, ewe	(Blaney Davidson *et al.* 2005; Cake *et al.* 2005; Jallali *et al.* 2005; Johnson *et al.* 2004; Quasnichka *et al.* 2005)
Genetically Modified		
ADAMTS-5 knockout	Mouse	(Glasson *et al.* 2005; Stanton *et al.* 2005)
Del1 transgenic	Mouse	(Morko *et al.* 2004)
Cathepsin K transgenic	Mouse	(Morko *et al.* 2005)
BMP receptor type 1a	Mouse	(Rountree *et al.* 2004)
Mig	Mouse	(Zhang *et al.* 2005)
Biglycan/fibromodulin knockout	Mouse	(Ameye *et al.* 2002; Wadhwa *et al.* 2005)
IL-6 knockout	Mouse	(de Hooge *et al.* 2005)
Chol+	Mouse	(Xu *et al.* 2005)
IL1β, IL1β converting enzyme, inducible NO synthase	Mouse	(Clements *et al.* 2003)

Drugs/ Supplements		
Aledronate	Rat	(Hayami *et al.* 2004)
PD-0200347	Dog	(Boileau *et al.* 2005)
S-34219	Guinea pig	(Sabatini *et al.* 2005)
Pioglatazone	Guinea pig	(Kobayashi *et al.* 2005)
Vitamin C	Guinea pig	(Kraus *et al.* 2004)

Table 1.1: Animal models used in the study of osteoarthritis. Adapted from Ameye and Young(2006)

1.5 The Role of Subchondral Bone in Osteoarthritis

1.5.1 Historical Perspective

The subchondral bone plate provides both mechanical and nutrient support for the overlying articular cartilage. Early work by Simon et al. (1972) and Radin et al. (1984) showed that thickening of the subchondral bone occurred in response to loading in both guinea pig and rabbit models. Radin and Rose (1986) subsequently suggested that the thickening of the underlying subchondral bone could occur as a result of healing microfractures, which in turn increased the stiffness of the subchondral bone, reducing the shock absorbing capacity of that joint and placing the overlying cartilage at increased risk of injury.

This hypothesis was questioned by the findings of two studies, one in guinea pigs (Layton et al. 1988) and the other using the anterior cruciate ligament transaction model in dogs (Dedrick et al. 1993). In these experiments, the subchondral bone was evaluated using microscopic computerised tomography and the overlying articular cartilage was assessed for morphological and biochemical changes. Although thickening of the trabecular bone and subchondral bone plate were evident as OA progressed, these changes did not appear to precede those in the articular cartilage.

1.5.2 Alterations of Subchondral Bone in OA – Diagnostic Imaging

The radiographic alterations of the subchondral bone in OA are well documented and described (Jacobson *et al.* 2008). However, developments in diagnostic imaging modalities have provided the opportunity to investigate OA at an earlier and more dynamic stage. Dieppe et al. (1993)

demonstrated that increased activity of subchondral bone, as shown by increased uptake of technetium-labelled bisphosphonate, not only preceded radiographic evidence of progression of OA, but also acted as a reliable prognostic indicator for progression of osteoarthritic changes. More recently, MRI has come into routine use and has been shown to be able to detect changes in cartilage, synovial membrane (Fernandez-Madrid et al. 1995; Ostergaard et al. 1997; Ostergaard et al. 1998; Pelletier et al. 2008), ligaments (Hill et al. 2005; Mohana-Borges et al. 2005) and subchondral bone (Bealle and Johnson 2000; Guymer *et al.* 2007; Raynauld *et al.* 2008).

Alterations in bone marrow signal on MRI have been described using a number of terms including bone bruises (Yao and Lee 1988), bone marrow oedema (Wilson *et al.* 1988), bone contusions (Stafford *et al.* 1986) and occult bone lesions (Labovitz and Schweitzer 1998). Although the term bone marrow oedema is widely used, its histological definition of replacement of bone marrow fat by material containing hydrogen ions in the form of water (Alanen *et al.* 1998) is not appropriate in every case. Histological abnormalities found in areas showing oedema-like signal patterns on MRI also include bone marrow fibrosis, necrosis and trabecular abnormalities with very little bone marrow oedema (Zanetti *et al.* 2000), such that the term bone marrow lesion has emerged and is considered more appropriate in many cases (Felson *et al.* 2001; Hunter *et al.* 2006; Roemer *et al.* 2009).

Correlations between increased uptake of radionucleotide on nuclear scintigraphy and bone marrow lesions on MRI have been demonstrated by Boegard et al. (1999) and McAlindon et al. (1991). Subsequently, in a longitudinal study, Felson et al. (2003) compared bone marrow lesions identified on MRI to progression of OA on follow-up radiography. These investigators found strong associations between bone marrow lesions and the progression of OA within the same joint compartment. Conversely, Pessis et al. (2003) demonstrated that absence of subchondral bone

abnormalities and bone marrow lesions on initial MRI assessment predicted absence of progression of chondropathy at follow-up at 1 year.

Using their model of subchondral bone impact, Lahm et al. (2005) demonstrated using MRI that immediately after impact there was intact cartilage and subchondral fractures represented by bleeding, microfractures and fragmented bone trabecules. Ligaments and menisci were shown to be intact. These MRI findings were supported by histological examination revealing microfractures of cancellous bone and weight-bearing trabeculae. In a further study (Lahm et al. 2006) the same group demonstrated that on follow-up MRI assessment six months after the initial trauma there was thinning, signal changes and surface interruptions in the cartilage above the former subchondral fractures.

1.5.3 Alterations of Subchondral Bone in Osteoarthritis- Mechanical Properties

Although subchondral bone thickening is a hallmark of OA, subchondral bone affected by OA has been shown to be hypomineralised as compared to age matched and young controls (Grynpas et al. 1991). Li and Aspden (1997) demonstrated that although osteoarthritic subchondral bone was significantly thicker than normal or osteoperotic subchondral bone, the stiffness and density of osteoarthritic subchondral bone were significantly less than that of normal samples. On examination of the composition of the subchondral bone, it was found that osteoarthritic samples had a significantly reduced fraction of mineral and an increased mass fraction of water.

The concept of hypomineralisation and altered biochemical composition of the matrix of osteoarthritic bone is important, as it explains the difference between the structural and material properties of bone. Apparent density is a structural property that may be defined as bone mass/total volume. Material density is defined as bone mass/bone volume. Apparent density can increase in response to either an increase in mineralisation of the tissue, or an increase in bone

volume. Material density can decrease with increased bone volume if the mineralisation of the tissue has decreased (Burr 2004). Therefore, it is apparent density which is seen radiographically as sclerosis, but this gives no indication as to the material density of that tissue.

1.5.4 Alterations of Subchondral Bone in Osteoarthritis - Bone Matrix Composition and Cellular Metabolism

It has been shown previously that subchondral bone from patients with OA has increased levels of alkaline phosphatase protein (Mansell et al. 1997) and mRNA expression (Truong et al. 2006). Levels of osteocalcin in osteoarthritic subchondral bone are also elevated, both at protein level (Gevers and Dequeker 1987; Raymaekers *et al.* 1992) and mRNA level (Kuliwaba *et al.* 2000; Pullig *et al.* 2000; Truong *et al.* 2006).

Alterations in the collagen metabolism have been investigated (Mansell and Bailey 1998) and it was found that type I collagen synthesis was increased in osteoarthritic subchondral bone as was the collagen content, although this collagen was significantly less mineralised than normal subchondral bone. This group then went on to describe how the ratio of α1 chains: α2 chains increased significantly in OA subchondral bone as compared to normal controls (Bailey et al. 2002) and concluded that the disorganised collagen matrix with reduced mineralisation found in osteoarthritic bone could be explained by an alteration in the osteoblast phenotype in osteoarthritic bone resulted in production of collagen type I homotrimer. Interestingly, this change in the ratio of collagen-I α1: collagen-I α2 has also been demonstrated in the gene expression of osteoarthritic subchondral bone (Truong *et al.* 2006). The importance of collagen type I in the pathogenesis of OA has recently been further demonstrated using the Brittle IV (Brtl) mouse, a knock-in model in which defective type I collagen is expressed in bone (Blair-Levy et al. 2008). Using this model, it was shown that initially mice had normal cartilage but decreased subchondral bone integrity. However by the second month of life, all Brtl mice had evidence of osteoarthritic changes within the articular cartilage.

In vitro, increased osteocalcin and alkaline phosphatase levels have also been shown in primary osteoarthritic osteoblasts, thus mimicking the *in vivo* situation (Hilal et al. 1998). Various *in vitro* co-culture systems have been used to investigate alterations in the phenotype of osteoblasts affected by OA, and how these altered cells may interact with cartilage. Westacott et al. (1997) demonstrated the ability of osteoblasts isolated from osteoarthritic subchondral bone to increase glycosaminoglycan release from cartilage, as compared to cells obtained from normal subchondral bone. It has also been shown that osteoblasts isolated from sclerotic areas of subchondral bone from joints affected by OA could initiate a change in chondrocyte phenotype shift towards hypertrophy (Sanchez *et al.* 2005b). Subsequently, the same authors also demonstrated that sclerotic subchondral osteoblasts could contribute to cartilage degradation by stimulating chondrocytes to produce more MMP-3 and MMP-13 and by inhibiting aggrecan synthesis (Sanchez *et al.* 2005a).

Although there has been a considerable research drive towards changes in subchondral remodelling with regards to alterations in the osteoblast phenotype, comparatively little data is available on the role of the osteoclast. However, it has been shown that aledronate, a nitrogen-containing bisphosphonate and a potent inhibitor of osteoclastic bone resorption, is able to inhibit subchondral bone sclerosis, osteophyte formation and articular cartilage degeneration in an anterior cruciate ligament transaction model of OA in the rat (Hayami et al. 2004). Further work is necessary to further describe the role of the osteoclast in OA, however it would appear that osteoclastic bone resorption, bone formation by osteoblasts and signals from both osteoblasts and osetoclasts may be important in the pathogenesis of subchondral bone disease, and consequently of OA (Karsdal et al. 2008).

Various studies have investigated the role of cytokines in remodelling of the subchondral bone in OA. Among the findings are that TGF-β has been found to be increased in osteoarthritic bone, and this may represent a stimulus for increased collagen synthesis (Mansell and Bailey 1998; Mansell *et al.* 2007). Interleukin-6 has been shown to play a role in modulation of various chemokines produced by osteoblasts in OA: IL-8, monocyte chemoattractant protein 1 (MCP-1) and chemokine (C-C motif) ligand 5 (previously known as RANTES) (Lisignoli et al. 2002). These authors concluded that as well as being target cells in bone, osteoblasts also acted as primary effector cells and were therefore able to mediate physiological and pathophysiological immune-related processes. Interleukin-6 and IL-1β have also been implicated in involvement in the crosstalk between subchondral bone and cartilage (Sanchez *et al.* 2005a).

1.6 Interaction between Subchondral Bone and Articular Cartilage

There is mounting evidence of alterations in the subchondral bone in OA, at gross, microscopic, biomolecular and cellular level. Various investigators have endeavoured to identify exactly if, and indeed how, these changes in subchondral bone can affect the overlying cartilage.

The first hypothesis is that of a mechanical effect. As discussed previously, Radin's original hypothesis was that increased stiffness in the subchondral bone led to cartilage degeneration (Radin and Rose 1986). This hypothesis has to a large extent been disproven, by various studies demonstrating that the subchondral bone is in fact less dense and less stiff in OA as compared to normal controls (Li and Aspden 1997). A more recent study by Day et al. (2001) showed that bone stiffness was decreased and bone quantity was increased in the presence of OA. They suggest that the reduction in tissue stiffness caused greater tissue deformation than was compensated for by increased bone volume, and that this led to a loss of normal mechanical equilibrium between cartilage and bone mechanical properties.

The second hypothesis regards a local biological effect of the abnormal osteoblasts on the overlying articular cartilage. As previously discussed, the altered phenotype of osteoblasts in OA has been well described, and these cells have been shown to secrete more alkaline phopsphatase, osteocalcin, IL-6, TGFβ1, insulin-like growth factor and prostaglandin PGE_2 than normal osteoblasts (Hilal *et al.* 1999; Hilal *et al.* 1998; Massicotte *et al.* 2002). In addition, *in vitro* osteoblasts obtained from the subchondral bone of osteoarthritic patients have been shown to have an effect on cartilage degradation and the phenotype of articular chondrocytes (Sanchez *et al.* 2005b; Westacott *et al.* 1997). Degradation of articular cartilage has been shown to be mediated to a large extent by the increased activity of tissue metalloproteinases (Cawston and Wilson 2006). It has been shown that MMPs-3 and -9; IL-1α, -8 and -10 and TNFα were upregulated in osteoarthritic subchondral bone (Hulejova et al. 2007) and suggested that these cytokines and MMPs may have an effect on adjacent cartilage.

It is generally accepted that cartilage loss in OA occurs from the surface down, therefore it would seem unlikely that bone cell-derived metalloproteinases could have an effect on cartilage loss (Westacott 2002). However, as was shown by Sanchez et al. *in vitro* (2005a, b), it would seem more plausible that cytokines produced by osteoblasts during subchondral bone remodelling could have an effect on the phenotype of overlying chondrocytes, with the finding that more superficial cartilage layers are affected first in OA being explained by differences in response to cytokines by chondrocytes at different levels of the articular surface (Westacott 2002).

How then do the cytokines gain access from the subchondral bone to the articular cartilage? This would seem impossible if the long-held view that adult cartilage does not receive blood supply and nutrition (and also therefore a potential source of delivery of cytokines) from the subchondral bone plate and relies solely on nutrition from synovial fluid, were true. Although the calcified cartilage has been described as being not normally vascular, if at all (Burr 2004), it has also been argued that

there is in fact not an absolute barrier to diffusion of nutrients between subchondral bone and articular cartilage (Imhof et al. 1999). The anatomy and distribution of vascular channels in the normal subchondral plate were investigated using scanning electron microscopy (Clark 1990) who found that although most of the capillaries resided within the subchondral bone, a minority of the subchondral bone vessels opened into the calcified articular cartilage and there were preceded by cells which appeared to be cutting into the articular cartilage. The importance of contact between the subchondral bone and the overlying articular cartilage, and further evidence that they function as a single unit was demonstrated in a study using osteochondral grafts in baboons. In those grafts where direct contact between the subchondral bone of the autograft and of the host were prevented by methylmethacrylate coating, cartilage degeneration was found to develop over a lengthy time period of 3 years (Malinin and Ouellette 2000).

It would appear that direct communication between the subchondral bone and articular cartilage are important for normal joint health, and that there are vascular channels between the subchondral bone and articular cartilage even in the healthy adult. Therefore, cross-talk between articular cartilage and its underlying subchondral bone as has been demonstrated *in vitro*, is not only possible, but also plausible *in vivo*.

1.7 Palmar Osteochondral Disease as a Model for Subchondral Bone Mediated Osteoarthritis

1.7.1 Pathogenesis of Palmar Osteochondral Disease

The clinical condition of pain associated with subchondral bone injury of the distal condyles of MC/MTIII is recognised in most racing breeds including Thoroughbreds, Standardbreds, Quarter Horses and Scandinavian Cold-Blooded Trotters (Ross and Dyson 2003). The condition has been previously referred to as traumatic osteochondrosis (Pool 1996), although we prefer the term palmar osteochondral disease (POD) (Barr *et al.* 2009) as this term avoids any confusion with the developmental condition of osteochondrosis dissecans.

Grossly the lesions are characterised as small, ovoid defects in the palmar or plantar articular surface of the condyle of approximate 2-4mm diameter (Pool 1996). Lesions are centred about 5-8mm proximal to the transverse ridge and may be located 3-15mm from the sagittal ridge on either the medial or lateral condylar surfaces (Pool 1996). Lesions appear to vary in severity. Early findings include a focus of bluish discolouration of the subchondral bone visible through grossly normal articular cartilage. More severe changes include physical disruption of the subchondral bone associated with varying degrees of pathology of the overlying articular cartilage. Ultimately there may be collapse of the subchondral bone with ulceration of the articular cartilage (Barr *et al.* 2009; Riggs 2006). In some cases these lesions have been reported to be associated with catastrophic condylar fracture (Krook and Maylin 1988).

The condition is of clinical as well as research importance. The pathological changes are clinically evident as a performance limiting lameness which may be bilateral or, in some cases, quadrilateral and as a result the affected horse may present with a poor action rather than overt lameness (Ross and Dyson 2003). The condition may be difficult to diagnose due to an inconsistent response to intra-articular analgesia and because only severe lesions are appreciable on routine radiography of the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints (Ross and Dyson 2003). More specialised radiographic views may aid diagnosis (O'Brien *et al.* 1981; Pilsworth *et al.* 1988) and specialised imaging modalities such as nuclear scintigraphy (Ross 1998), computed tomography (Byron and Goetz 2007; Morgan *et al.* 2006) and magnetic resonance imaging (Zubrod *et al.* 2004) are extremely useful in diagnosis of POD. Visualization of the predilection site for POD lesions on the palmar/plantar aspect of the condyles of MC/MTIII is physically impossible during routine arthroscopic examination of the MCP/MTP joints. Consequently, many lesions remain undiagnosed until the later stages of disease, by which time the changes are irreversible and will result in chronic joint disease.

1.7.2 Reasons for using POD as a Model of Osteoarthritis

As outlined in Table 1.1, various experimental models for investigating OA *in vivo* using the horse have been described including; osteochondral chip fragmentation of the radial carpal bone (Frisbie *et al.* 2002), an instability model of the metacarpophalangeal joint (Simmons *et al.* 1999), synovitis induced by lipopolysaccharide (Todhunter *et al.* 1996) and chemical injury of articular cartilage using sodium monoiodoaceate (Gustafson *et al.* 1992). These techniques are useful in re-creating experimental forms of equine OA however have drawbacks as the resulting osteoarthritic process is often more aggressive than the naturally occurring disease. The ideal model of OA would follow a more natural pattern of disease and also allow investigation of the early stages of pathology.

POD has been described previously as a model of overload arthrosis, with the equine palmar metacarpal condyle providing a reliable site at which microfracture and subchondral bone failure may occur, although attempts to re-create the disease in treadmill exercise horses resulted in mild lesions only (Norrdin *et al.* 1998). Given that POD can manifest in a range of severities of disease from mild, early-stage disease, through moderately affected joints, to late, end-stage disease as outlined in Chapter 5 (Post Mortem Evaluation of Palmar Osteochondral Disease (Traumatic Osteochondrosis) of the Metacarpo-/Metatarsophalangeal Joint in Thoroughbred Racehorses) it is considered that the condition can be utilised as a naturally occurring *ex vivo* condition to allow the investigation of OA at various stages of pathology. Further, since the disease process is thought to originate in the subchondral bone as opposed to the overlying hyaline articular cartilage, the disease has the potential to act as a model for subchondral bone driven joint disease and also to provide information as to the relationship between hyaline articular cartilage and subchondral bone disease and how these two closely related but anatomically separate tissues interact within the joint.

1.8 Aims and Hypotheses of the Thesis

Utilising knowledge from the previously published literature as outlined above, the aims of the PhD project were to:

1) set up and validate an *in vitro* co-culture system of equine osteoblasts and chondrocytes
2) utilise this co-culture system to investigate interactions between equine osteoblasts and chondrocytes *in vitro* at gene and metabolome level
3) investigate further the pathogenesis of a naturally occurring equine osteochondral disease process utilising data derived from post mortem examination, histology examination and gene expression experimental studies.

It was hypothesised that:

1) osteoblasts could be isolated from equine SCB, undergo reliable proliferation and differentiation and respond to IL1β such that the osteoblasts showed an osteoarthritic phenotype *in vitro*
2) co-culture of equine osteoblasts pre-treated with IL1β with cartilage explants from joints unaffected by OA would result in an altered chondrocyte phenotype and evidence of cartilage matrix degradation
3) there would be detectable differences in the metabolic profiles of conditioned culture media from various equine osteoblast and equine osteoblast and chondrocyte co-culture models, and that useful biomarkers could be identified after analysis of the spectra
4) POD would have a high prevalence at *post mortem* examination of a population of intensively raced Thoroughbreds with a predilection sites for these pathologies. Further, that there would be an association between POD and other pathologies affecting the distal condyles of MC/MTIII and a correlation between prevalence of POD and pathologies of the dorsodistal aspect of MC/MTIII, suggestive of MCP/MTP joint hyperextension

5) POD would have a characteristic and well-defined histological appearance and that the OOCHAS scoring system would be more reliable in analysis of cartilage pathology in samples of condyles affected by POD

6) there would be increases in gene expression of matrix proteinases in SCB, hyaline articular cartilage (HAC) and synovial membrane affected by POD and alterations in expression of genes involved in matrix synthesis in SCB and HAC

7) there would be correlations between expression of genes in the various articular tissues (SCB, HAC and synovial membrane)

8) there would be an increase in expression of non-collagenous matrix protein genes in early disease (Grade 1 POD) with a reduction in expression of non-collagenous matrix protein genes in later/more severely affected SCB (Grades 2 and 3)

9) there would be an increase in expression of genes involved in osteoclastic activity in SCB affected by POD, particularly more severe grades of POD (Grades 2 and 3), as compared to SCB unaffected by POD (Grade 0).

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Culture of Equine Osteoblasts and Response to Interleukin 1β

2.1 Introduction

The *in vitro* culture of osteoblast-like cells is a long standing technique in cell biology and bone metabolism research (Robey and Termine 1985). Osteoblast-like culture models may be based on immortalised cell lines, either derived from retroviral delivery of cDNA (Heath *et al.* 1989) or from sarcomas (Heremans *et al.* 1978; McAllister *et al.* 1971); primary cultures obtained through explant culturing (Beresford *et al.* 1984; Beresford *et al.* 1986); or bone marrow cultures where osteoblasts are differentiated from precursors in the marrow stroma (Beresford 1989).

While immortalised cell lines have advantages in terms of ease of availability in human studies, they do not necessarily provide a representative model of the osteoblastic phenotype (Clover and Gowen 1994). In the equine, a supply of suitable cadaver material is more readily available such that obtaining primary cultures of osteoblast-like cells is easily achievable. However, although there are numerous descriptions of studies on osteogenic cells isolated from both human and laboratory animals, there is relatively much less published literature on culture of equine osteoblasts. What has been reported is related to viability and osteoprogenitor potential of bone grafts (McDuffee and Anderson 2003; McDuffee *et al.* 2006) rather than to the basic biology of equine osteoblast-like cells *in vitro*. Therefore, in order to develop a co-culture system for equine osteoblasts and cartilage explants, it was important to validate a system whereby equine osteoblasts could undergo reliable proliferation and differentiation. It was also important to identify suitable culture media for the differentiation and treatment steps and to ascertain whether once differentiated the osteoblasts were remaining viable in response to changes in various culture media which would be used for subsequent co-culture experiments (See Chapter 3). The response of equine osteoblasts to treatment with interleukin 1β (IL1β) had also to be investigated as this has not been described previously in the literature.

It was hypothesised that osteoblasts could be isolated from equine SCB, undergo reliable proliferation and differentiation and respond to IL1β such that the osteoblasts showed an osteoarthritic phenotype *in vitro*.

2.2 Materials and Methods

For all materials and methods, reagents were obtained from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

2.2.1 Experimental Design

Experiment 1: Choice of Osteogenic Media

Primary osteoblasts isolated from bone explants from one donor were cultured for a further 12 days in one of 3 culture media, with medium changes every 2-3 days:

1) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B,10mM HEPES, 2% Ultroser G, 10^{-8}M $1,25(OH)_2$vitamin D_3, 50µg/ml ascorbic acid and 2mM proline (Sanchez *et al.* 2005)
2) DMEM supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone (Jonsson *et al.* 1999)
3) DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (negative control)

Differentiation of osteoblasts was assessed by staining with Alizarin Red and Von Kossa, quantitative real time PCR (RT-qPCR) analysis of expression of osteocalcin (*BGLAP*) and cellular alkaline phosphatase assay. Confirmation that cells were not adipocytes was assessed by negative staining for Oil Red O.

Experiment 2: Do osteoblasts show alterations in gene expression in response to IL1β, and if so what is the optimum concentration of IL1β to drive a degradative phenotype?

Primary osteoblasts from bone explants from one donor were isolated and on reaching confluence were differentiated in osteogenic medium for 10 days (DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone). Cells were cultured without IL1β, or with 10ng/mL IL1β or 100ng/mL IL1β and were harvested at 24 hours, 48 hours or 72 hours.

Outcome was measured by RT-qPCR analysis of expression of *BGLAP*, *MMP3* and *COL1A2*.

Experiment 3: Do osteoblasts remain differentiated and retain altered gene expression if IL1β treatment is applied for 24hours then removed?

Primary osteoblasts from bone explants from three donors were isolated and on reaching confluence were differentiated in osteogenic medium for 10 days (DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone). After differentiation, cells were cultured for 24 hours in DMEM serum free medium either with or without treatment with 10ng/ml IL1β. After 24 hours of IL1β treatment, cells were washed in Hank's Balanced Salt Solution (HBSS) before culturing in DMEM serum free medium for 72 hours. Cells were harvested at 24, 48 and 72 hour time points.

Outcome was measured by RT-qPCR analysis of expression of *BGLAP*, *MMP3* and *COL1A2*; cellular alkaline phosphatase activity and staining for Alizarin Red.

Experiment 4: Does pre-treatment of osteoblasts with IL1β have an effect on gene expression on a panel of genes involved in matrix synthesis, proteinases and inhibitors of proteinases?

Primary osteoblasts were isolated from bone explants from 3 separate donors and differentiated as before. After differentiation, cells were cultured for 24 hours in serum free medium with or without the addition of 10ng/ml IL1β. After IL1β pre-treatment, cells were washed and cultured for a further 72 hours in serum-free medium. At the end of this period cells were harvested in Tri-reagent®.

Outcome was measured by RT-qPCR analysis of expression of biglycan (*BGN*), *BGLAP*, *COL1A2*, *COL2A1*, *MMP1*, *MMP3*, *MMP13*, *ADAMTS4*, *ADAMTS5* and *TIMP3*.

Experiment 5: In longer cell culture systems, is viability and maintenance of osteoblast differentiation supported by the addition of serum/ITS+ to the culture medium?

Primary osteoblasts were isolated from bone explants and differentiated as before. After differentiation, cells were cultured for 24 hours in one of the 4 cell culture media listed below, either with or without treatment with 10ng/ml IL1β.

1) DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (serum free medium)
2) DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+ (1% ITS+ medium)
3) DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B and 1% FBS (1% FBS medium)
4) DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B and 10% FBS (10% FBS medium)

After 24 hours of IL1β treatment, cells were washed in Hank's Balanced Salt Solution (HBSS) before culturing in the same culture medium as before. Cells were harvested at T=0 (directly after

osteogenic differentiation); T=72h (+/- IL1β pre-treatment) and T=144h (+/- IL1β pre-treatment). The time point of 72 hours was chosen as this would be the length of the gene expression co-culture and the time point of 144 hours was chosen as this would be the length of the GAG release co-culture.

Outcome was measured as live/dead viability staining, Alizarin Red and Von Kossa staining of the osteoblasts.

2.2.2 Tissue Collection

Material was collected either from horses euthanased at The University of Liverpool Equine Hospital for reasons unrelated to orthopaedic disease/osteoarthritis and where owners had given informed consent for use of samples from their horse, or from healthy horses euthanased at a local abattoir. All material was harvested within 12 hours of the horse's death. Although exact signalment of abattoir cases was not available, all subjects were mature and all joints from which samples were obtained were grossly free of osteoarthritis. Subchondral bone was obtained from either the metacarpo- or metatarsophalangeal joint. Cartilage was removed from the distal condyles and after removal of the superficial calcified cartilage, the subchondral bone plate was removed from the palmar/plantar aspect of both lateral and medial distal condyles by sterile dissection with a chisel, such that small fragments of bone with dimensions approximately 3mm x 3mm were obtained.

2.2.3 Subchondral Osteoblasts in Monolayer Culture

Based on the protocols of Hilal et al. (1998) and Sanchez et al. (2005) the small pieces of subchondral bone were sequentially incubated in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) at 37°C, initially with 0.5mg/ml hyaluronidase type IV S for 20 minutes then with 0.6mg/ml collagenase IA for 4 hours. The digested bone pieces were then washed in Hank's Balanced Salt Solution (HBSS) before culturing in T-75 flasks in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml amphotericin B. Medium was changed twice weekly until cells were observed to have migrated from the bone explants and had reached

confluence. At this point cells were collected by trypsinisation, seeded at 20,000 cells/cm^2 in 12 well plates and grown for 12 days in an osteogenic differentiation medium.

Osteoblasts were cultured for a further 10-12 days in one of 3 differentiation media as detailed in experiment 1, with medium changes every 2-3 days. On the basis of the results of this experiment, thereafter cells were differentiated by culturing for 10-12 days in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone, with media changes every 2-3 days.

2.2.4 Alizarin Red, Von Kossa's and Oil Red O Staining

Alizarin Red and Von Kossa's Staining were performed to ascertain differentiation of the osteoblasts and confirm osteoblast phenotype. Oil Red O staining was performed to identify any adipogenic differentiation. For all stains, media was removed from the wells and cells were washed twice in phosphate buffered saline (PBS) prior to fixing of the cells with 4% formaldehyde for 10 minutes. Cells were washed twice in ultra-pure water before application of the stain. For the Alizarin Red stain, 1% Alizarin Red S was applied for 5 minutes then washed twice in ultra pure water. For the Von Kossa stain, 1% silver nitrate was applied to the cells and then cells were placed under ultraviolet light for 40 minutes. Cells were washed in distilled water then treated with 3% sodium thiosulphate for 5 minutes. For the Oil Red O stain, a stock of 0.5% Oil Red O in propan-2-ol was diluted 3 in 5 in ultra pure water, then applied to the cells and incubated at room temperature for 1 hour. Staining was assessed by examination by microscope (Nikon Eclipse TS100; Nikon, Kingston Upon Thames, UK) and digital pictures were obtained.

2.2.5 Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was quantified in the cellular fraction of the osteoblast culture. Media was removed at the end of the co-culture period (72h). Cells were washed in PBS then lysed in 1ml 0.2% Triton X-100 and the Triton-X/cell lysate mixture was collected. Cell extract (5µL) was incubated with 195µL of a 10mM p-Nitrophenyl phosphate working solution (BioAssay Systems). A standard solution of tartrazine was used as a calibrator, according to the kit's instructions. In the presence of alkaline phosphatase, p-Nitrophenyl phosphate is converted to p-nitrophenol and inorganic phosphate, with the rate of the reaction being directly proportional to the enzyme activity. The absorbance of p-nitrophenol was measured at 405nm on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland)at room temperature at t=0 and t=4 minutes. ALP activity of the sample is measured according to the kit's instructions as:

$$\text{ALP (IU/L)} = \frac{(\text{OD sample t} - \text{OD sample 0}) \times \text{Reaction Volume}}{(\text{OD calibrator} - \text{OD } H_2O) \times \text{Sample Volume} \times t} \times 40.4$$

Alkaline phosphatase activity was normalised to total protein content of the cellular fraction.

2.2.6 Total Protein Assay

Total protein content of the osteoblast cellular fraction collected in 0.2% Triton X-100 was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Thermo Scientific, Rockford, Illinois) according to the manufacturer's instructions. This technique is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. Cu^{1+} is detected in the presence of bicinchoninic acid (BCA) by the chelation of 2 molecules of BCA with one Cu^{1+} ion resulting in a purple-coloured reaction product. The assay was measured on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland) at 570nm relative to standards of albumin with known protein concentration.

2.2.7 Live/Dead Staining

Media was removed from osteoblasts in monolayer and cells were washed in sterile PBS. Twenty µL of 2mM ethidium homodimer (EthD-1) was added to 10ml of sterile PBS, and to this mixture 5µL of 4mM calcein AM stock solution was added according to the kit's instructions (Live/Dead Viability/Cytotoxicity Kit for mammalian cells; Invitrogen Ltd, Paisley, UK). This gave a final solution of 2µM calcein AM and 4µM EthD-1 which was added directly to the wells of the 12-well plate containing the washed cells. Cells were incubated for 30 minutes at room temperature in the dark. After incubation, cells were examined under a fluorescence microscope (Nikon Eclipse TS100; Nikon, Kingston Upon Thames, UK). Live cells were visualised using an excitation filter of 460-500nm and emission of 510-560nm. Dead cells were visualised using an excitation filter of 540-580nm and emission of 600-660nm. Digital images were obtained and stored.

2.2.8 Quantitative Real-Time PCR

2.2.8.1 RNA extraction and Reverse Transcription

Osteoblasts were harvested in Tri-Reagent (Ambion, Applied Biosystems, Warrington, UK). RNA extraction was performed using a standard chloroform and ethanol extraction followed by the RNeasy (Qiagen, Crawley, UK) column technique, incorporating a DNase treatment stage. RNA was quantified using a Nanodrop (Thermo Scientific, Wilmington, USA) and 1µg of RNA or the maximum volume for the reaction (12.4µL) where RNA concentration was < 80.6ng/µL, was used as the template for the reverse transcriptase reaction. cDNA strands were generated from the RNA in a 25µL volume reaction using Random Primers (Promega, Southampton, UK), RT buffer, 10mM of dNTPs, M-MLV RT enzyme and RNase inhibitor.

2.2.8.2 Primer Preparation

Equine specific PCR primers were designed by obtaining equine RNA and DNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/). Where the equine sequence was unavailable, a multiple species alignment was performed (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and primers designed where there was sequence homology across species. Exon boundaries were identified (http://www.ensembl.org/index.html) and where possible primers were designed to amplify across exon-intron boundaries to allow discrimination of genomic DNA and cDNA. As this was not possible in all cases, the DNase treatment stage was incorporated in the RNA extraction technique as above. Primers were designed based on the sequences acquired using Primer Express software (Applied Biosystems) or Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer efficiencies were validated using a standard curve derived from equine cDNA using a 10-fold dilution series with 5 measuring points. A dissociation curve was included in validation to ensure primers were specific and amplified only one product. Primer efficiency is optimal at a slope of -3.32. The primers used for the target and housekeeping genes and their efficiencies are as shown in Table 2.1.

Gene	Primer Sequence	Accession Code	Efficiency
GAPDH	F: GCATCGTGGAGGGACTCA R: GCCACATCTTCCCAGAGG	AF157626	-3.32
ADAMTS4	F: CAGCCTGGCTCCTTCAAAAA R: CCGCAGGAATAGTGACCACAT	NM_001111299	-3.23
ADAMTS5	F: ACCGATCCTGCAGTGTCACA R: CTGCTCATGGCGAAAAGATTT	EU025851	-3.13
MMP 1	F: GGTGAAGGAAGGTCAAGTTCTGAT R: AGTCTTCTACTTTGGAAAAGAGCTTCTCT	NM_001081847	-3.36
MMP-3	F: TCTTGCCGGTCAGCTTCATATAT R: CCTATGGAAGGTGACTCCATGTG	NM_001082495	-3.63
MMP 13	F: CTGGAGCTGGGCACCTACTG R: ATTTGCCTGAGTCATTATGAACAAGAT	NM_001081804	-3.51
TIMP-3	F: CTGCAACTTCGTGGAGAGGT R: ACTCGTTCTTGGAGGTCACG	NM_001081870	-3.54
Collagen I α2	F: GCACATGCCGTGACTTGAGA R: CATCCATAGTGCATCCTTGATTAGG	XM_001492939	-3.31
Collagen II α1	F: TCAAGTCCCTCAACAACCAGATC R: GTCAATCCAGTAGTCTCCGCTCTT	NM_001081764	-3.21
Biglycan	F: TCACCTTCCAGCCCCTAGAGT R: AGAAGCAGCCCCTCCTCAA	NM_001081839	-3.71
Osteocalcin	F: TCAACCCAGACTGTGACGAG R: CAGCTAGGGACGATGAGGAC	XM_001915727	-3.80

Table 1: Primer sequences used for quantitative real-time PCR
F= Forward primer, R= Reverse primer

2.2.8.3 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 25μL volume using SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) and 300nM primer concentration and processed by 7300 Real Time PCR system (Applied Biosystems, Warrington, UK). Conditions for amplification were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C for primer annealing and elongation. This was followed by a dissociation stage for 15 seconds at 95°C, 30 seconds at 60°C and 15 seconds at 95°C to ensure the presence of a single amplicon. Data was analysed using SDS software (Applied Biosystems). PCR products were measured and normalised against GAPDH as a housekeeping gene.

2.2.9 Data Analysis

Data was plotted and tested for normality using the Kolmogrov-Smirnov test, then logged as required (Minitab v.15; Minitab Inc., Pennsylvania). Where appropriate, mixed effects linear regression (SPlus v6.1; TIBCO Software Inc, California) was used to test for significant differences between groups while allowing for the clustering of samples within donor. Significance was set at $P<0.05$ unless multiple comparisons were made on the same sample (i.e. gene expression data), where the P-value was adjusted using Sidak's formula (Sidak 1967):

$$\alpha_{PT} = 1 - (1-\alpha_{PF})^{1/c}$$

where α is the probability of making a Type I error, α_{PT} is the α per test, α_{PF} is the α per family of tests and c is the number of comparisons. Therefore if we assume that the required α_{PF} is 0.05, then:

$$\alpha_{PT} = 1 - (1-0.05)^{1/c}$$

2.3 Results

2.3.1 *Experiment 1: Choice of Osteogenic Media*

Cell staining (Alizarin Red, Von Kossa, Oil Red O), qRT-PCR for osteocalcin and cellular alkaline phosphatase assay were performed in triplicate from one donor. Results for cell staining in the various differentiation media are as shown in Table 2.2. Images of the cell staining are as shown in Figures 2.1-2.6. Gene expression of osteocalcin was increased in cells differentiated in medium containing β-glycerophosphate, ascorbic acid and dexamethasone (Figure 2.7) as compared to the other media. Alkaline phosphatase assays showed markedly increased cellular levels of alkaline

phosphatase in cells cultured in both osteogenic differentiation media as compared to the negative control (Figure 2.8).

Stain	**Culture Media**: DMEM, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B plus:		
	10mM HEPES, 2% Ultroser G, 10^{-8}M 1,25$(OH)_2$vitamin D_3, 50µg/ml ascorbic acid and 2mM proline	10% FBS, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone	10% FBS (negative control)
Alizarin Red	negative	positive	negative
Oil Red O	negative	negative	negative
Von Kossa	mildly positive	positive	negative

Table 2.2: Response to staining after differentiation of cells in various culture media

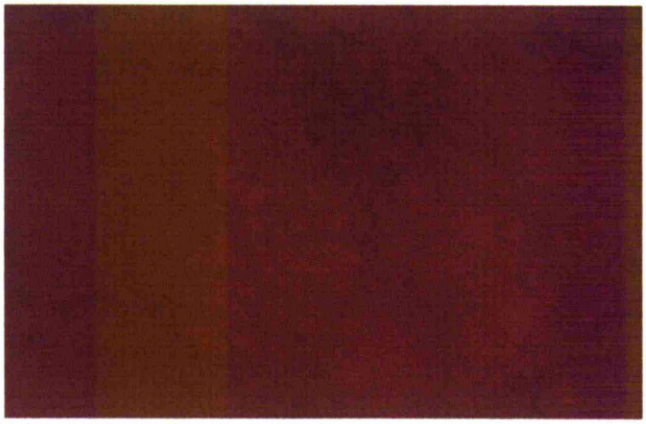

Figure 2.1: Positive (red) staining for Alizarin Red in osteoblasts differentiated in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone

Figure 2.2: Negative staining for Alizarin Red in osteoblasts differentiated in DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B,10mM HEPES, 2% Ultroser G, 10^{-8}M 1,25$(OH)_2$vitamin D_3, 50µg/ml ascorbic acid and 2mM proline

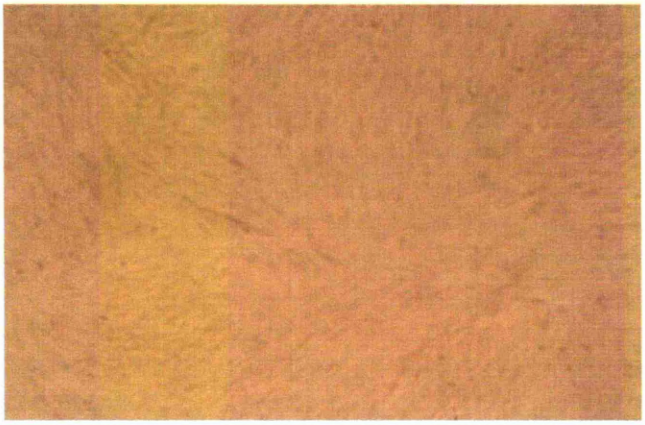

Figure 2.3: Negative staining for Alizarin Red in osteoblasts differentiated in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (negative control)

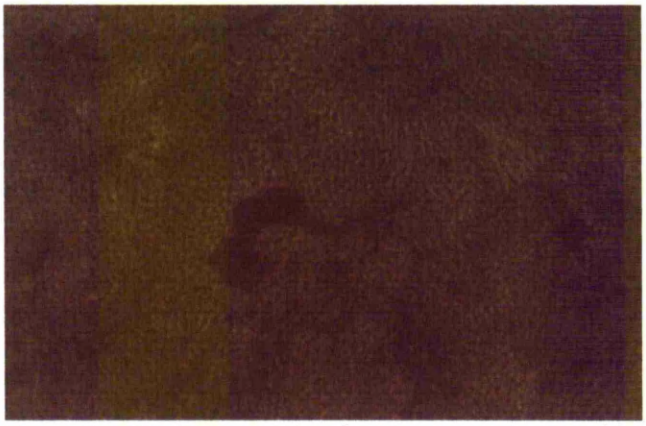

Figure 2.4: Positive staining for Von Kossa in osteoblasts differentiated in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone

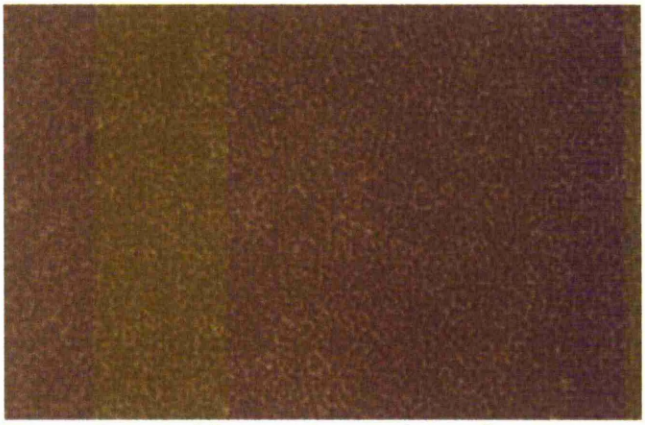

Figure 2.5: Mildly positive staining for Von Kossa in osteoblasts differentiated in DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B,10mM HEPES, 2% Ultroser G, 10^{-8}M 1,25(OH)$_2$vitamin D_3, 50µg/ml ascorbic acid and 2mM proline

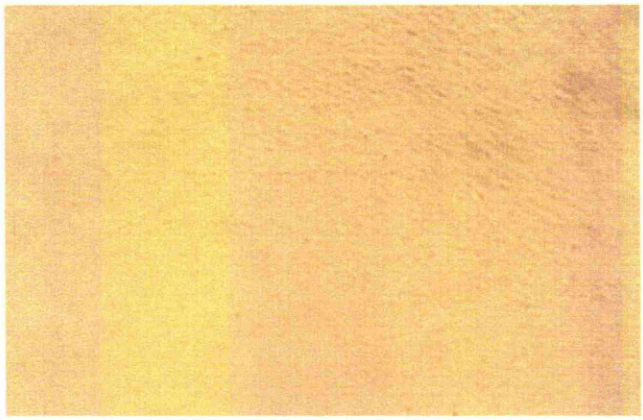

Figure 2.6: Negative staining for Von Kossa in osteoblasts differentiated in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (negative control)

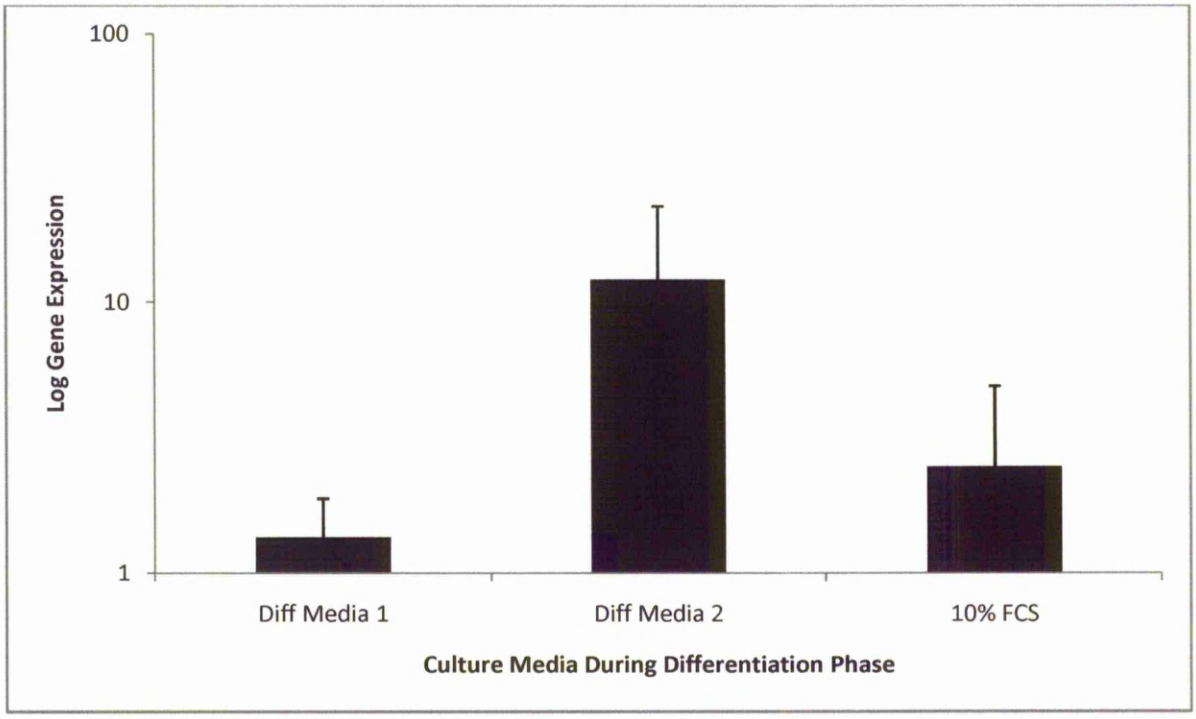

Figure 2.7: Osteocalcin expression in osteoblasts after 10 days in various differentiation media. Diff Media 1: DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B,10mM HEPES, 2% Ultroser G, 10^{-8}M 1,25(OH)$_2$vitamin D$_3$, 50µg/ml ascorbic acid and 2mM proline; Diff Media 2: DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone; 10% FCS: DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (negative control)

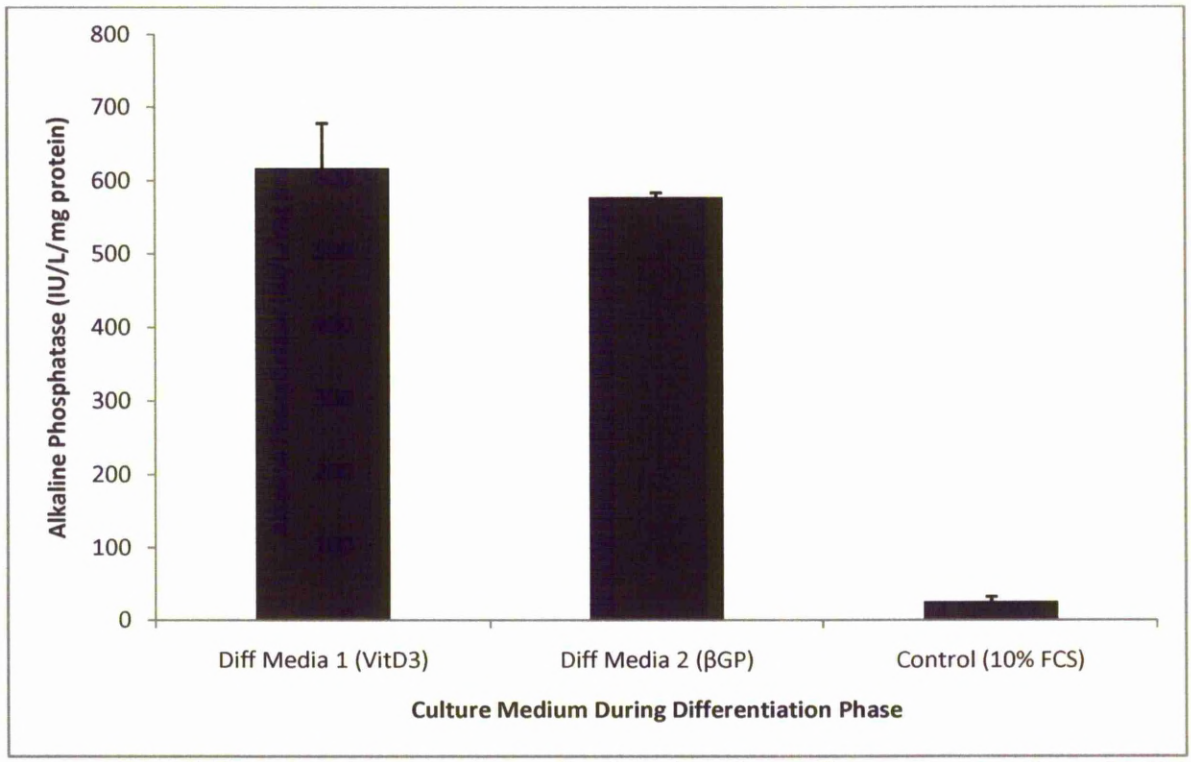

Figure 2.8: Alkaline Phosphatase content of cells cultured in Differentiation Media 1; DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B,10mM HEPES, 2% Ultroser G, 10^{-8}M 1,25(OH)$_2$vitamin D$_3$, 50µg/ml ascorbic acid and 2mM proline; Differentiation Media 2: DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone; 10% FCS: DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B (negative control)

It was concluded that using culture medium 2 (DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β-glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone) resulted in the most reliable differentiation of primary osteoblast-like cells for use in future experiments.

2.3.2 ***Experiment 2: Do osteoblasts show alterations in gene expression in response to IL1β, and if so what is the optimum concentration of IL1β?***

Quantitative RT-PCR to measure expression of *BGLAP*, *MMP3* and *COL1A2* for osteoblasts treated with 0ng/mL, 10ng/mL and 100ng/mL and harvested at times 0, 24, 48 and 72 hours were performed

in triplicate from cells grown from one donor. Because only one donor was used in this experiment, statistical analysis was not performed. Results are as shown in Figures 2.9-2.11.

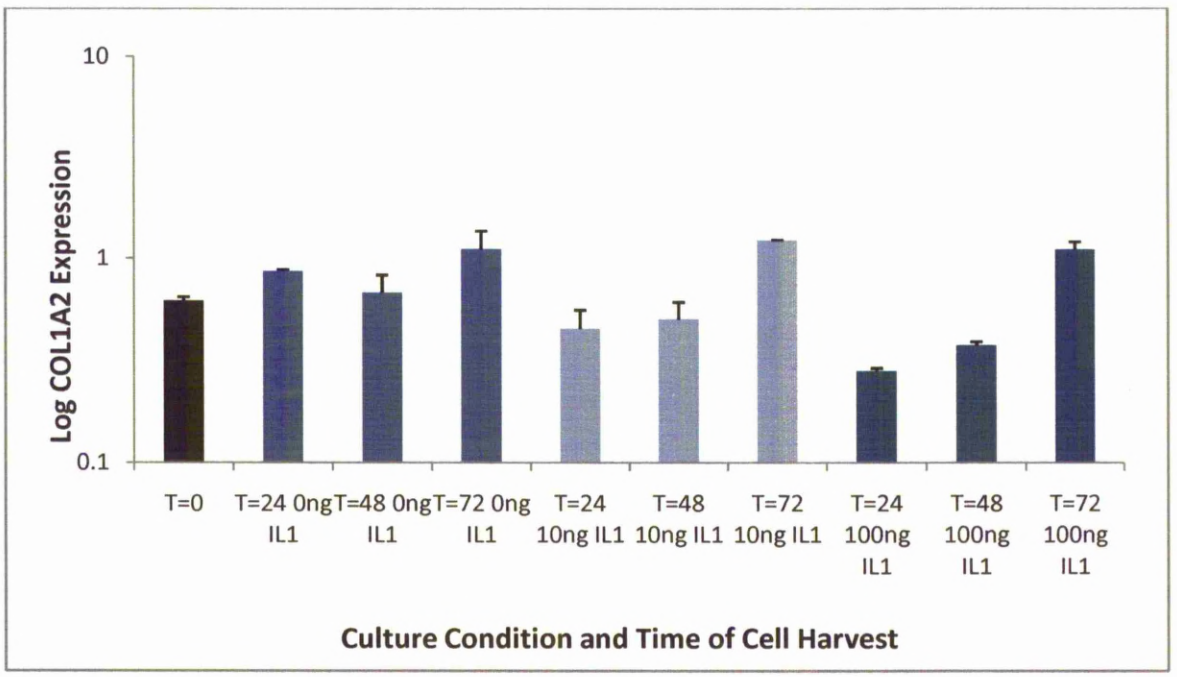

Figure 2.9: COL1A2 expression relative to GAPDH as a housekeeping gene (shown as a log value) at times 0, 24, 48 and 72 hours without IL1β treatment and with 10ng/mL and 100ng/mL IL1β added to the culture media

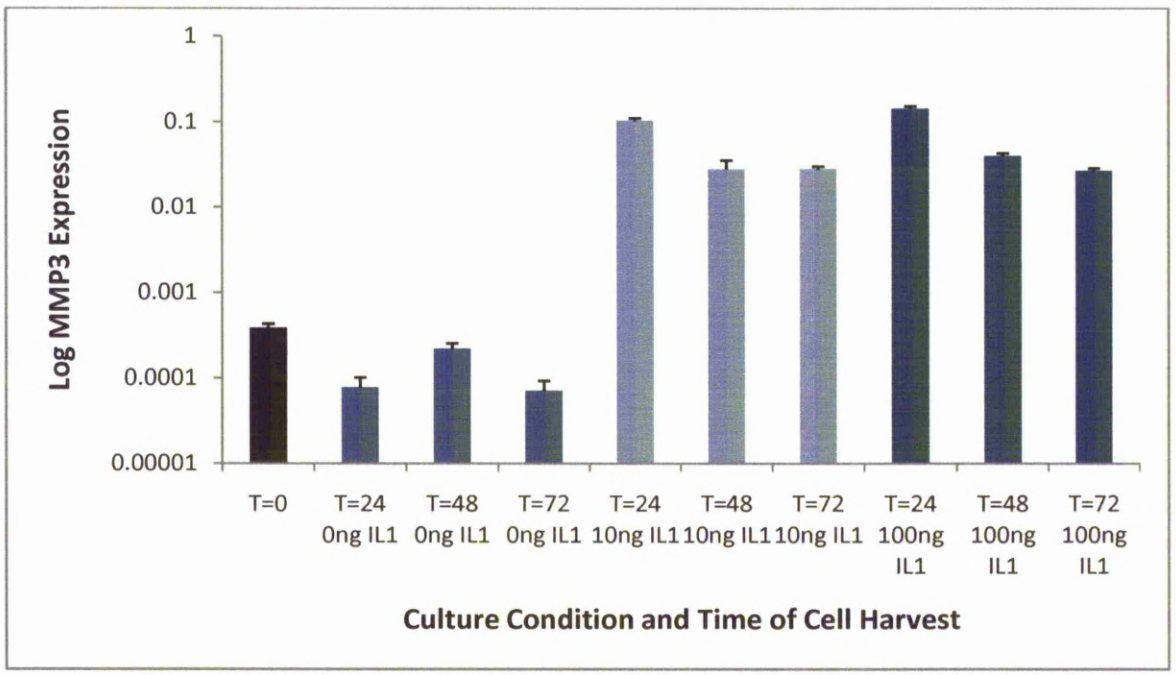

Figure 2.10: MMP3 expression relative to GAPDH (shown as a log value) at times 0, 24, 48 and 72 hours without IL1β treatment and with 10ng/mL and 100ng/mL IL1β added to the culture media.

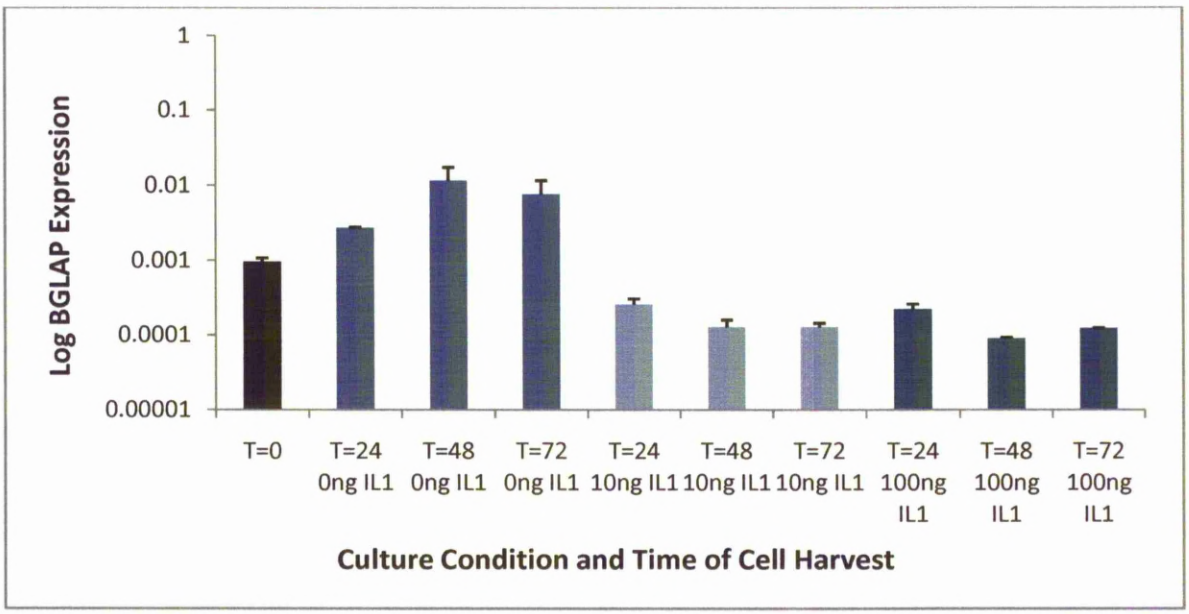

Figure 2.11: Osteocalcin expression relative to GAPDH (shown as a log value) at times 0, 24, 48 and 72 hours without IL1β treatment and with 10ng/mL and 100ng/mL IL1β added to the culture media.

MMP3 expression was up-regulated after treatment with both 10ng/mL and 100ng/mL IL1β at all time points. *BGLAP* expression was decreased after osteoblasts were treated with both 10ng/mL and 100ng/mL IL1β at all time points. *COL1A2* expression was decreased after treatment with IL1β at time points 24 and 48 hours, with a more pronounced decrease in expression at the higher concentration of 100ng/mL. By 72 hours, expression of *COL1A2* was similar in all treatment groups (without IL1β, 10ng/mL IL1β and 100ng/mL IL1β).

From these results it was concluded that the lower concentration of 10ng/mL IL1β should be used for stimulation of osteoblasts towards an osteoarthritic phenotype in subsequent experiments.

2.3.3 ***Experiment 3: Do osteoblasts remain differentiated and retain altered gene expression if IL1β treatment is applied for 24hours then removed?***

Previously it was shown that there were alterations in expression of various genes in osteoblasts treated with 10ng/mL IL1β for 72 hours. For development of the co-culture system, it was necessary to investigate how equine osteoblasts would respond to 24 hours of IL1β treatment at 10ng/mL followed by removal of this stimulus.

Experiments were performed in triplicate on primary osteoblasts obtained from 3 separate donors. Gene expression of *MMP3*, *COL1A2* and *BGLAP* are as shown in Figures 2.12-2.14, expressed as fold difference in gene expression normalised to the osteoblasts not pre-treated with IL1β at 24 hours. The Sidak corrected level of significance was calculated as P≤0.02. Where differences in gene expression are significant between treatment groups at each time point, these are shown on the graph.

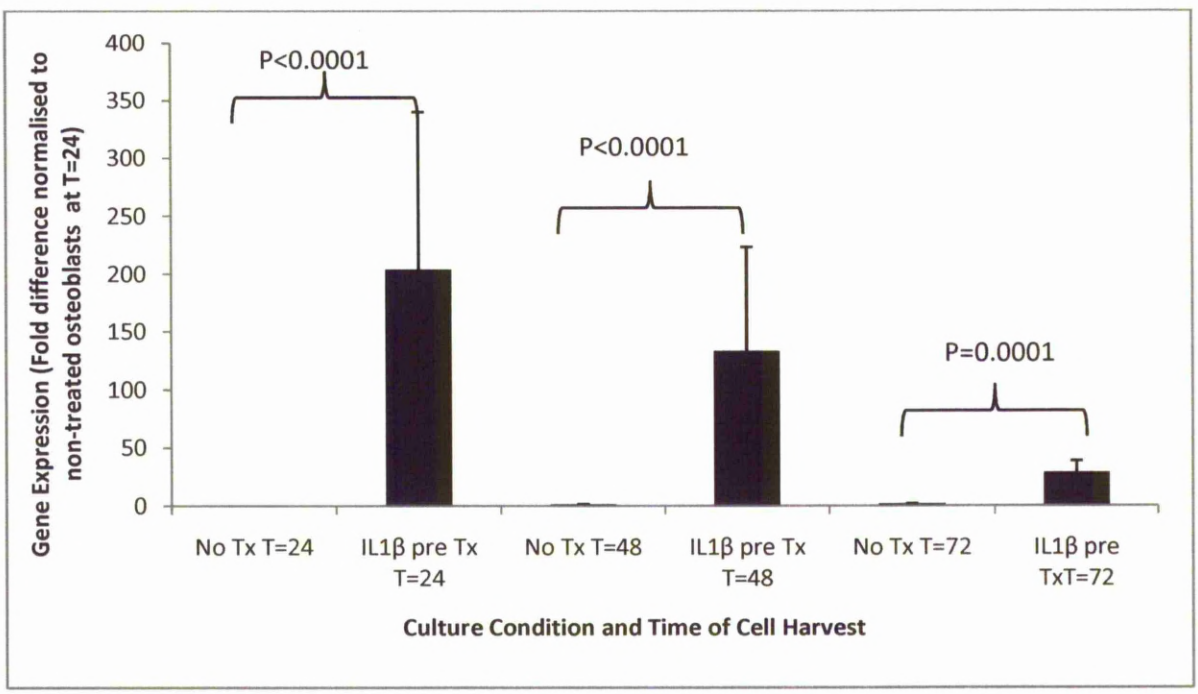

Figure 2.12: MMP3 expression from 3 donors in osteoblasts pre-treated or not with IL1β for 24 hours, then washed and cultured in serum free media for a further 72 hours maximum. Culture times are expressed as time after washing of cells and removal of IL1β or washing of non-treated controls. Gene expression is expressed as fold increase in gene expression normalised to the osteoblasts not pre-treated with IL1β at T=24 hours. Error bars indicate standard error.

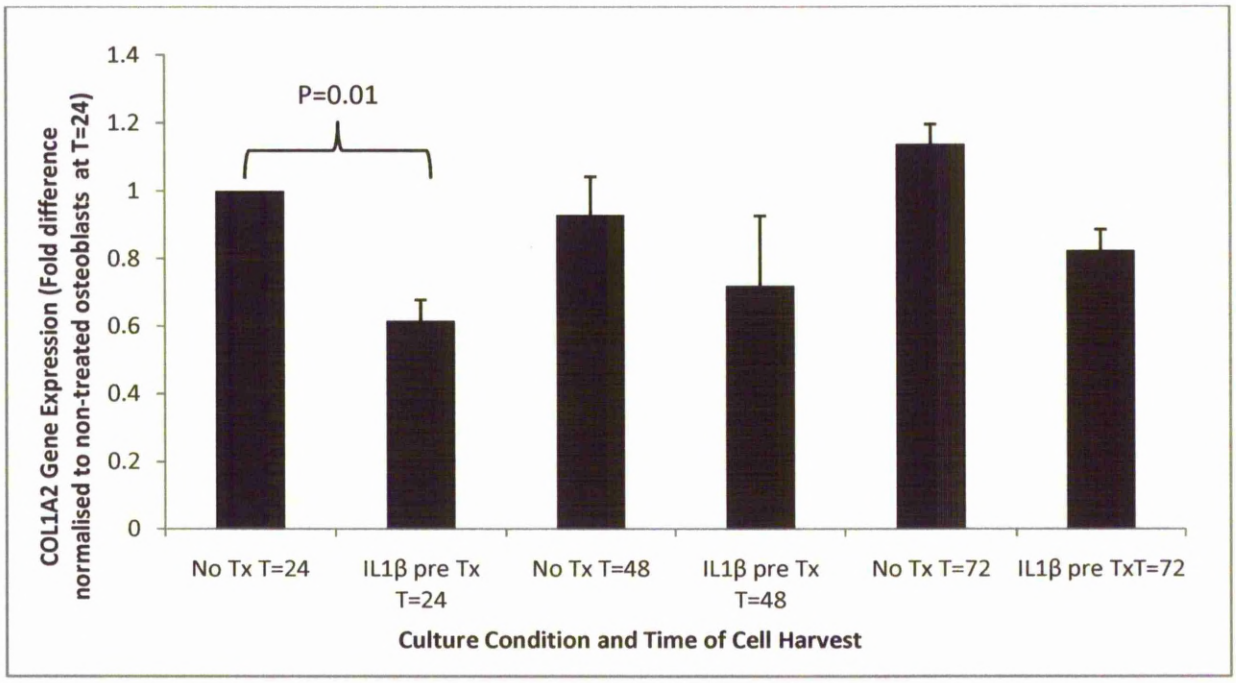

Figure 2.13: Collagen-I expression from 3 donors in osteoblasts pre-treated or not with IL1β for 24 hours, then washed and cultured in serum free media for a further 72 hours maximum. Culture times are expressed as time after washing of cells and removal of IL1β or washing of non-treated controls. Gene expression is expressed as fold increase in gene expression normalised to the osteoblasts not pre-treated with IL1β at T=24 hours. Error bars indicate standard error.

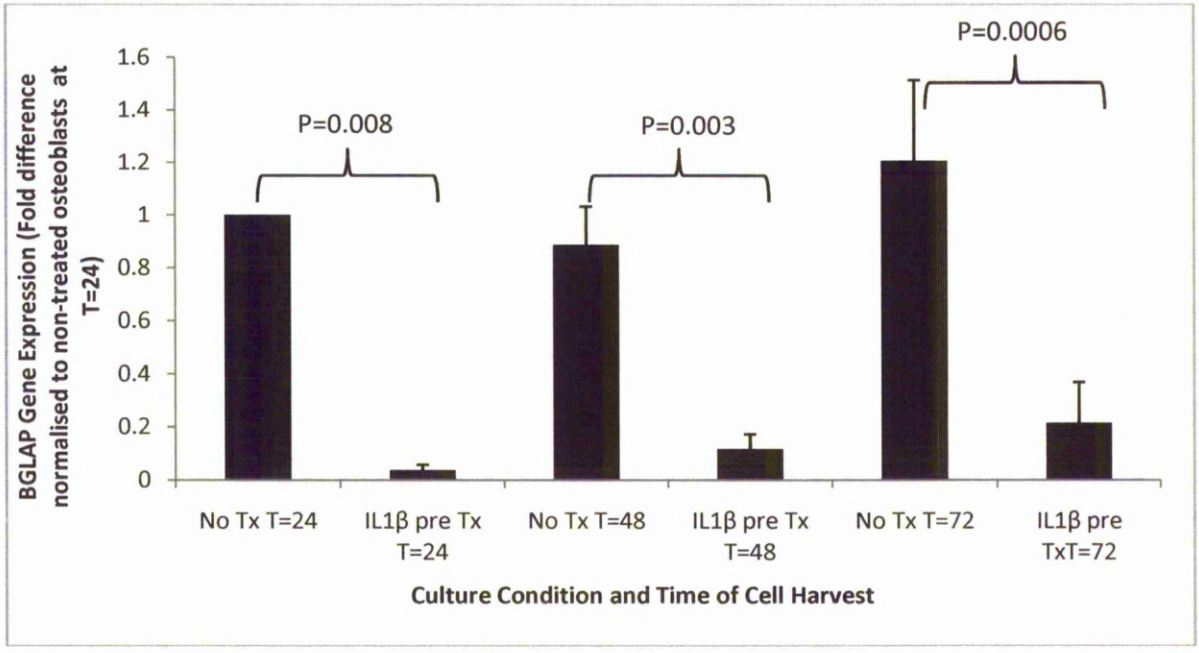

Figure 2.14: Osteocalcin expression from 3 donors in osteoblasts pre-treated or not with IL1β for 24 hours, then washed and cultured in serum free media for a further 72 hours maximum. Culture times are expressed as time after washing of cells and removal of IL1β or washing of non-treated controls. Gene expression is expressed as fold increase in gene expression normalised to the osteoblasts not pre-treated with IL1β at T=24 hours. Error bars indicate standard error.

MMP3 expression was significantly increased in the IL1β pre-treated osteoblasts at all time points measured. *COL1A2* expression was significantly decreased in IL1β pre-treated cells at 24 hours, but there was no significant difference between groups in *COL1A2* expression at 48 or 72 hours. *BGLAP* expression was significantly decreased in IL1β pre-treated cells at all time points.

Results of alkaline phosphatase assays are as shown in Figure 2.15. There were no significant differences in alkaline phosphatase levels between IL1β pre-treated and non IL1β pre-treated osteoblasts at any time point. Thus pre-treatment of osteoblasts with IL1β did not appear to have an effect on alkaline phosphatase content of the cells.

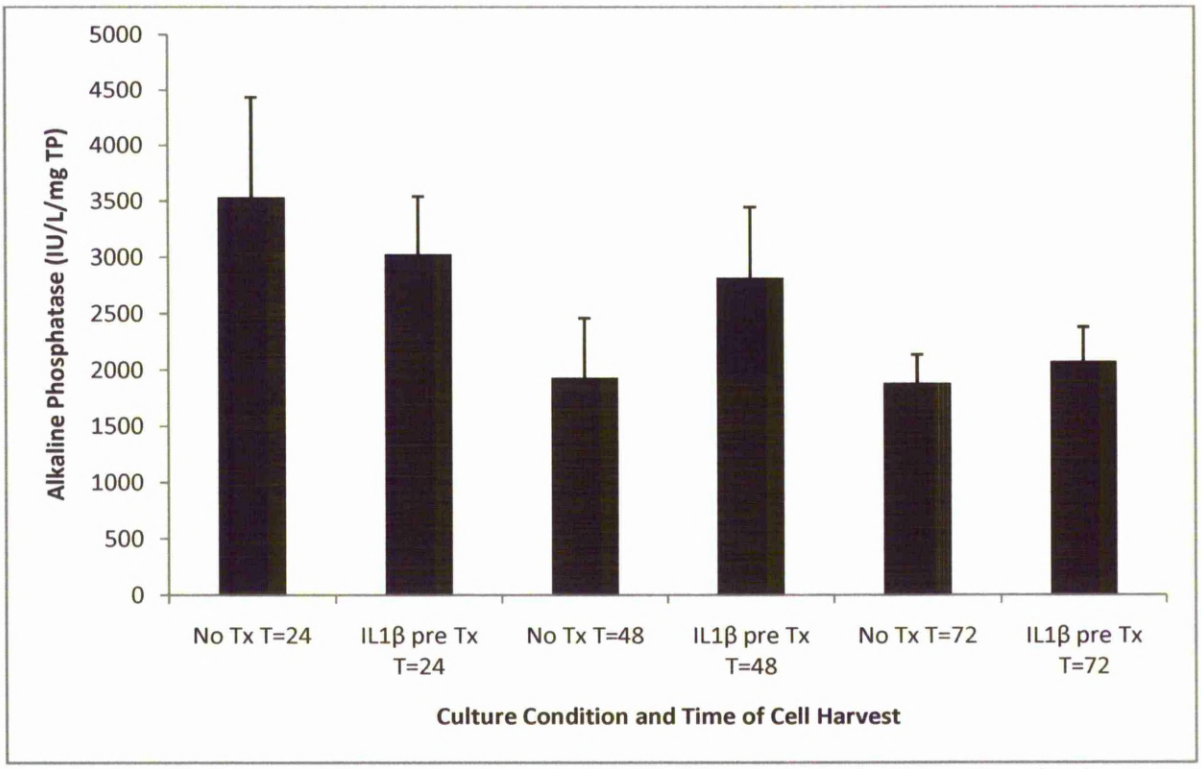

Figure 2.15: Alkaline phosphatase content of the osteoblast cellular fraction normalised to total protein content of samples from 3 donors expressed as IU/L/mg TP. Error bars indicate standard error.

Osteoblasts which were pre-treated with IL1β showed an apparent reduction in Alizarin Red staining at 24 hours (Figure 2.16) as compared to osteoblasts which had not been pre-treated (Figure 2.17). By 48 hours however, the difference between IL1β pre-treated and non pre-treated osteoblasts was much less evident (Figures 2.18 and 2.19).

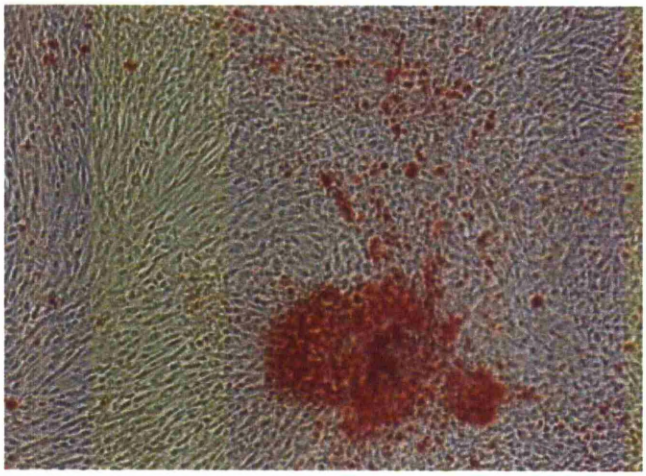

Figure 2.16: Alizarin Red staining of osteoblasts pre-treated with IL1β at 24 hours of culture in serum free media

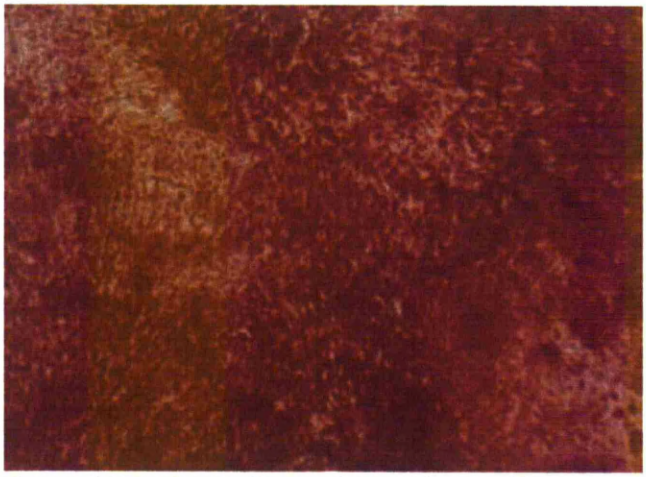

Figure 2.17: Alizarin Red staining of osteoblasts not pre-treated with IL1β at 24 hours of culture in serum free media

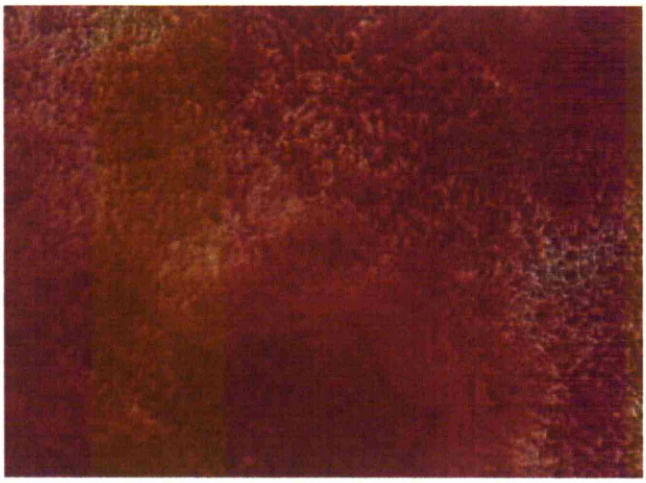

Figure 2.18: Alizarin Red staining of osteoblasts pre-treated with IL1β at 48 hours of culture in serum free media

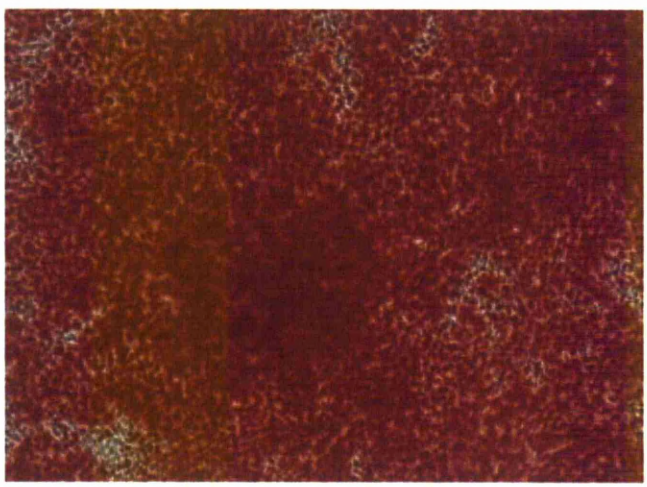

Figure 2.19: Alizarin Red staining of osteoblasts not pre-treated with IL1β at 48 hours of culture in serum free media

2.3.4 ***Experiment 4: Does pre-treatment of osteoblasts with IL1β have an effect on gene expression on a panel of genes involved in matrix synthesis, proteinases and inhibitors of proteinases?***

Experiments were performed in triplicate on primary osteoblasts obtained from 3 separate donors. Differentiated osteoblasts were either pre-treated with 10ng/mL or not pre-treated with IL1β for 24 hours, washed, then cultured for a further 72 hours in serum free medium. Expression of a panel of genes of interest (*MMP1*, *MMP3*, *MMP13*, *ADAMTS4*, *ADAMTS5* and *TIMP3*) in osteoblasts pre-treated or not with IL1β are as shown in Figure 2.20. Expression of each gene is shown for osteoblasts pre-treated with IL1β as a fold difference to the expression of that gene in differentiated

osteoblasts grown in monolayer but not pre-treated with IL1β. The Sidak corrected level of significance was calculated as P≤0.009.

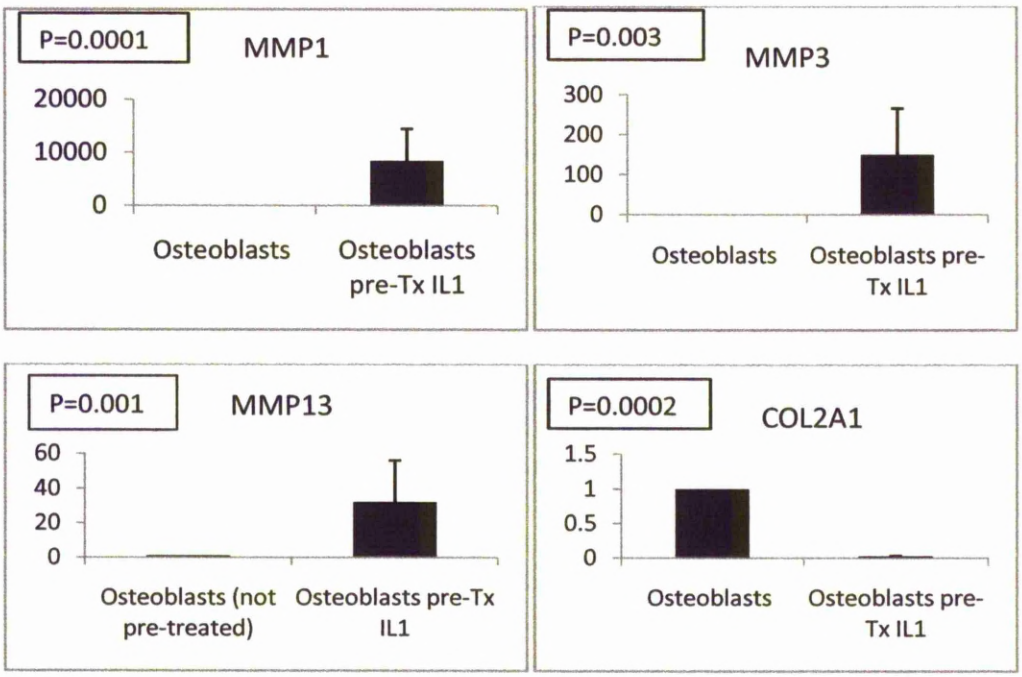

Figure 20: Gene expression with significant differences in osteoblasts pre-treated with IL1β as compared to osteoblasts not treated with IL1β. Values for gene expression are shown as a fold differences compared to expression in the untreated osteoblasts. Significance of differences in expression are shown on each graph.

In the IL1β pre-treated osteoblasts there was a significant increase in expression of *MMP1* (P=0.0001), *MMP3* (P=0.003), *MMP13* (P=0.001). There was a significant decrease in expression of *COL2A1* (P=0.0002) in the IL1β pre-treated osteoblasts. There was no significant difference in expression of *COL1A2*, *BGN*, *BGLAP*, *ADAMTS4*, *ADAMTS5* or *TIMP3* between the IL1β pre-treated and non pre-treated osteoblasts.

3.5 ***Experiment 5: In longer cell culture systems, is viability and maintenance of osteoblast differentiation supported by the addition of serum/ITS+ to the culture medium?***

Response of osteoblasts to live/dead, Alizarin Red and Von Kossa staining with and without pre-treatment with IL1β at 72 hours and 144 hours in DMEM, DMEM with 1% ITS+, DMEM with 1% FCS

and DMEM with 10% FCS are as shown in Figures 2.21-2.36. Primary osteoblasts were grown from one donor.

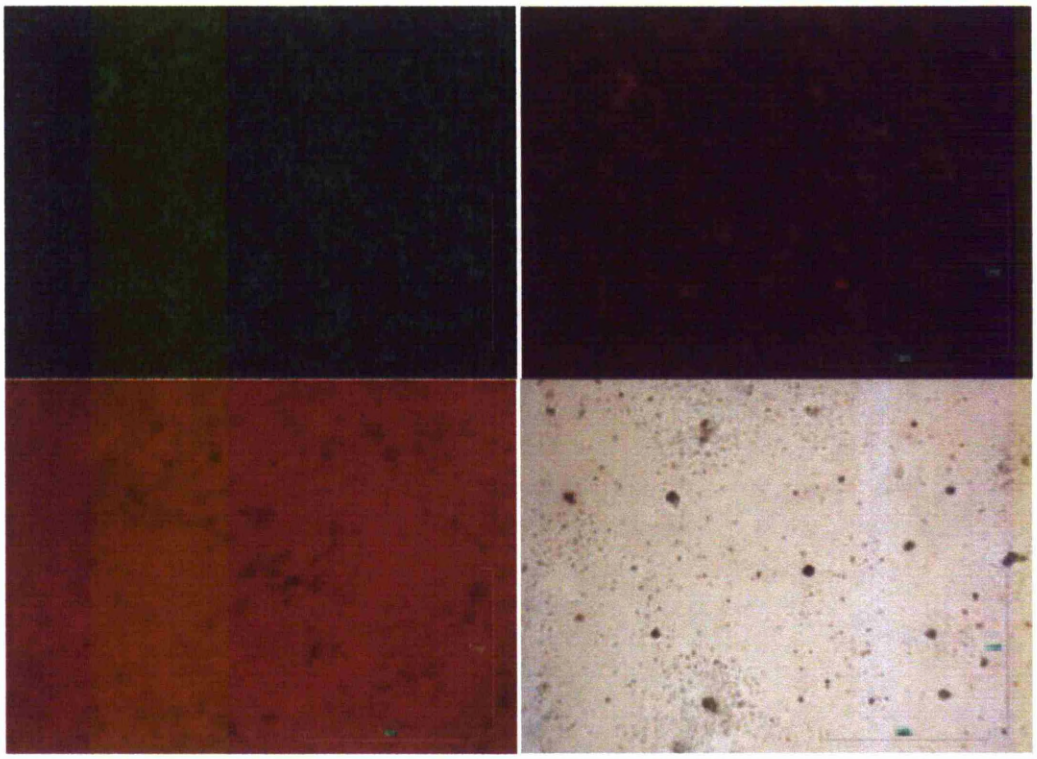

Figure 2.21: Non IL1β pre-treated osteoblasts cultured for 72 hours in serum free DMEM. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

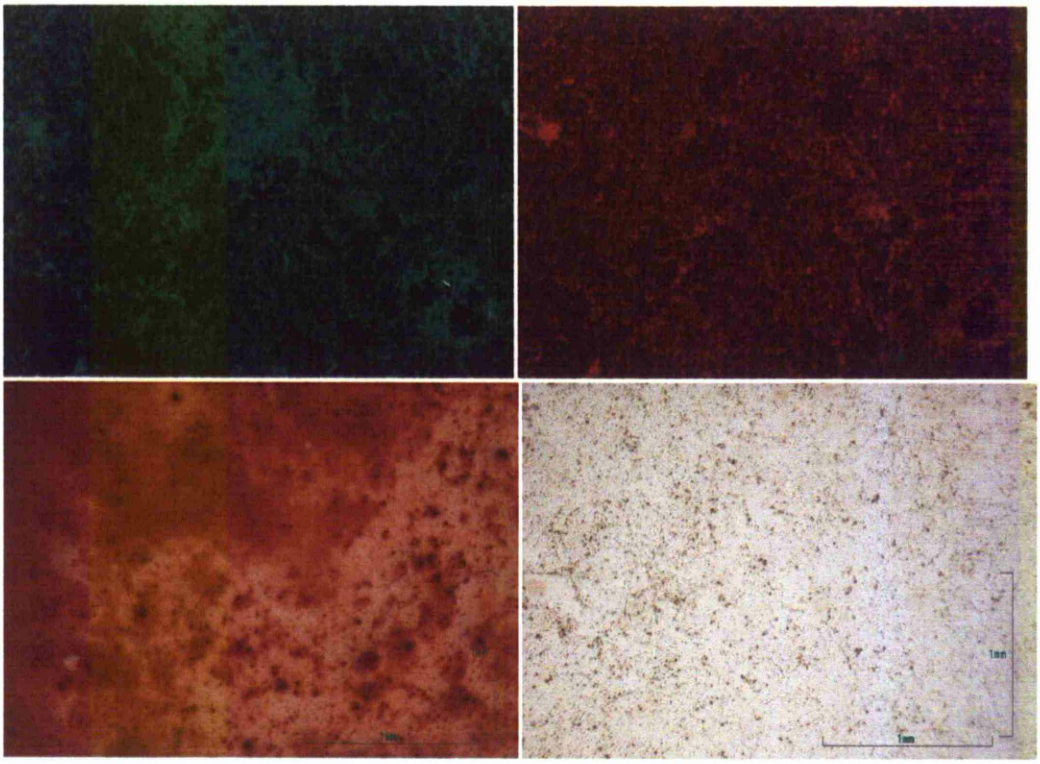

Figure2. 22: IL1β pre-treated osteoblasts cultured for 72 hours in serum free DMEM. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

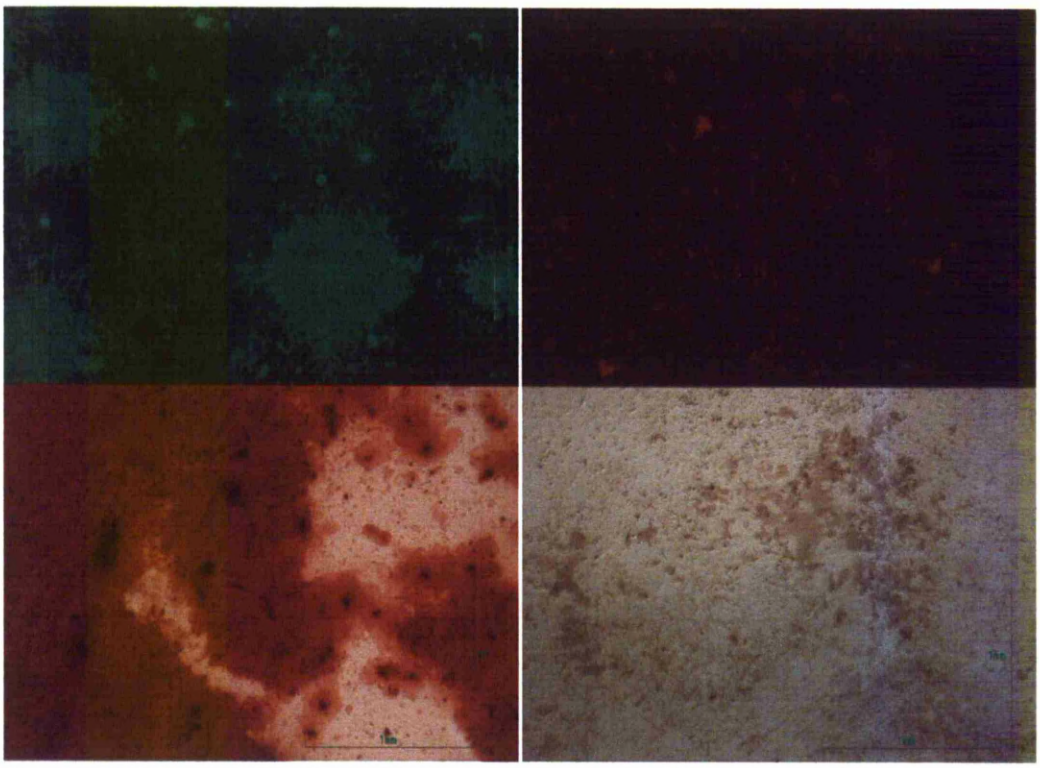

Figure 2.23: Non IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 1% ITS+. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

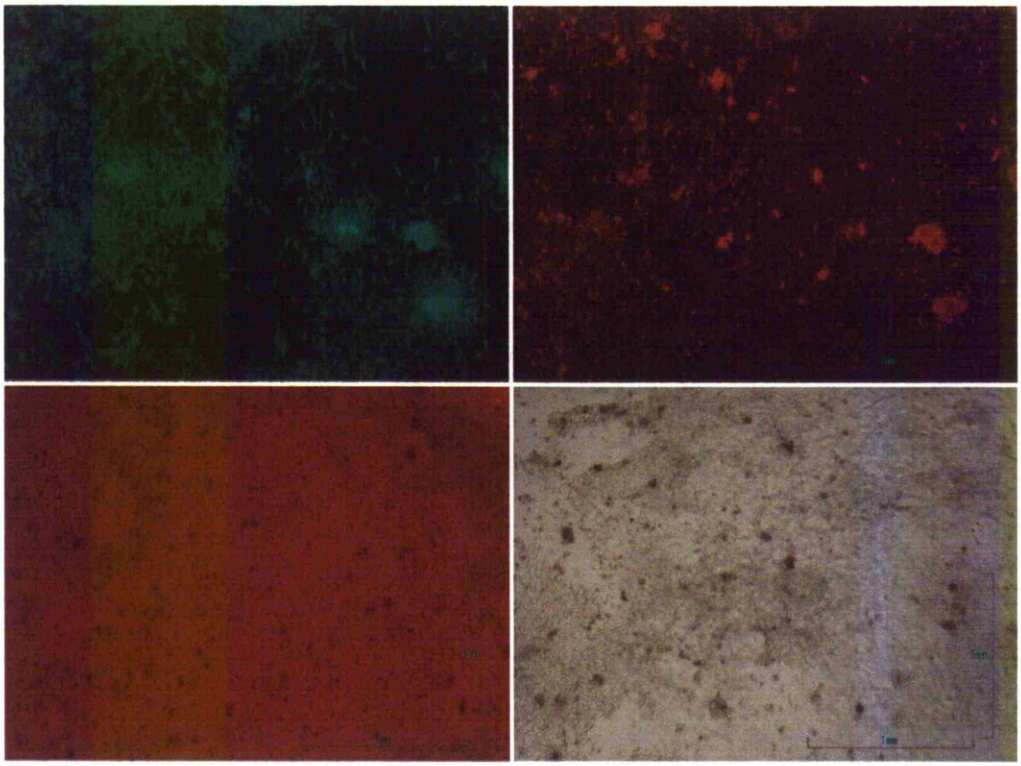

Figure 2.24: IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 1% ITS+. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

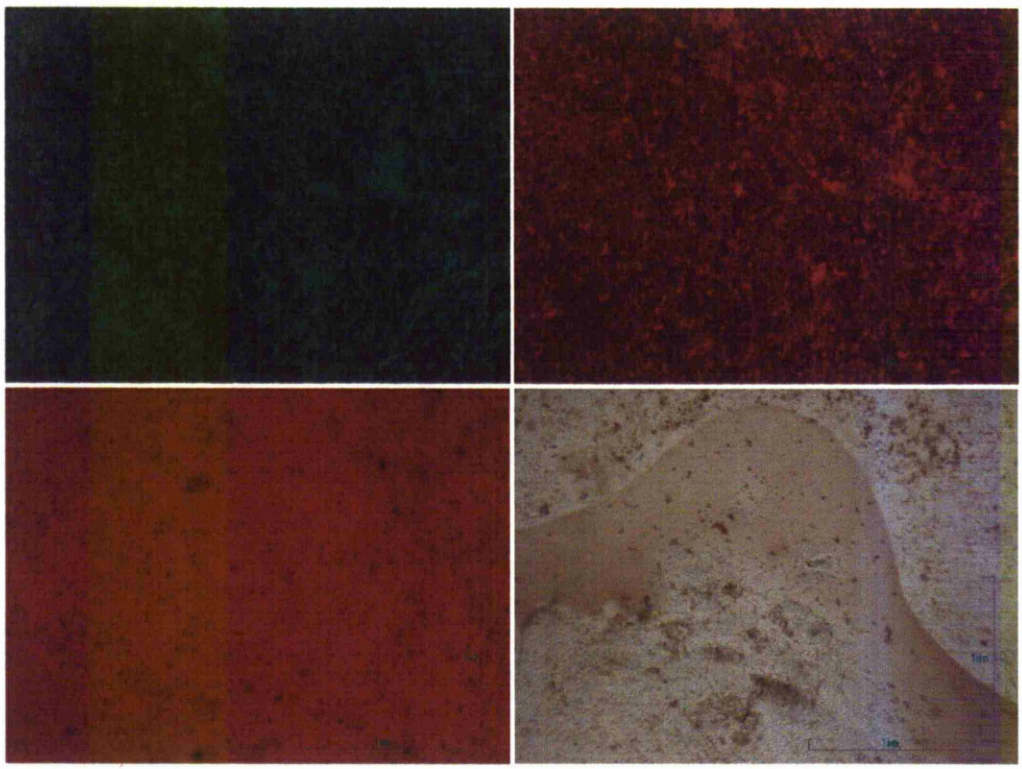

Figure 2.25: Non IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 1% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

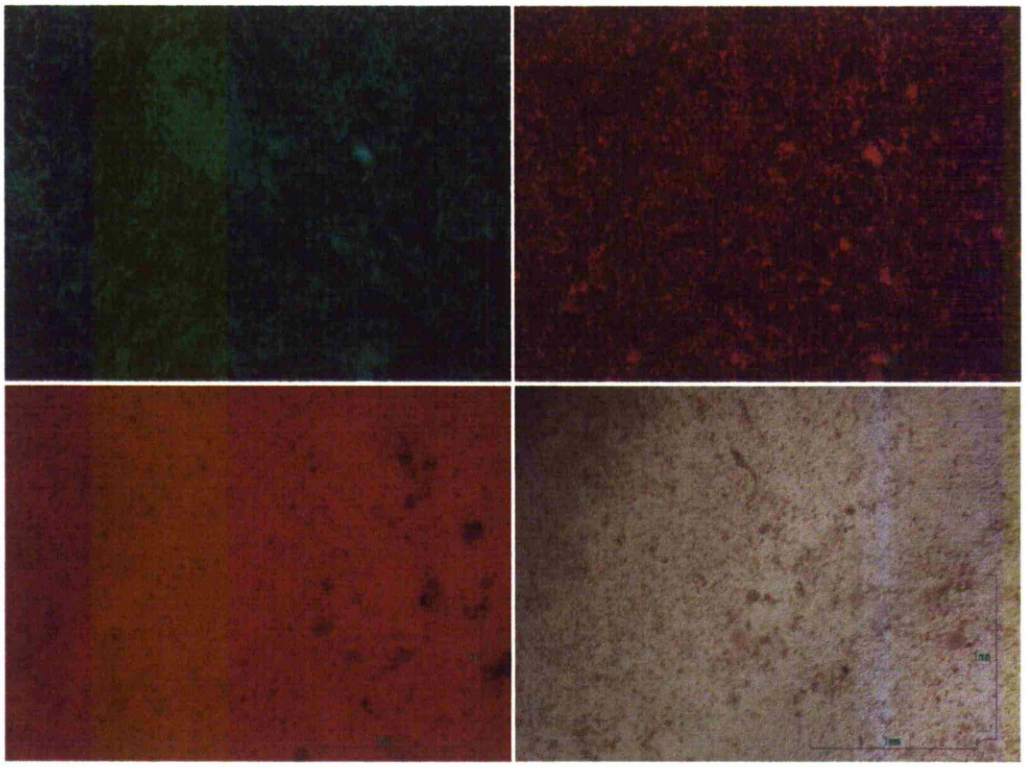

Figure 2.26: IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 1% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

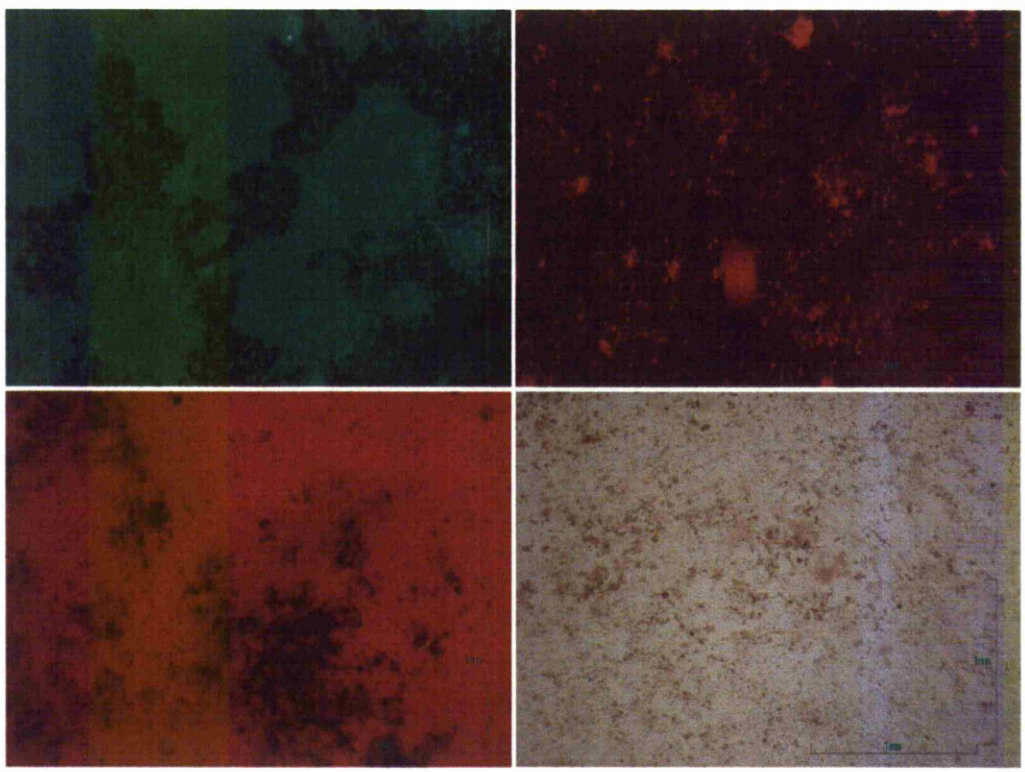

Figure 2.27: Non IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 10% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

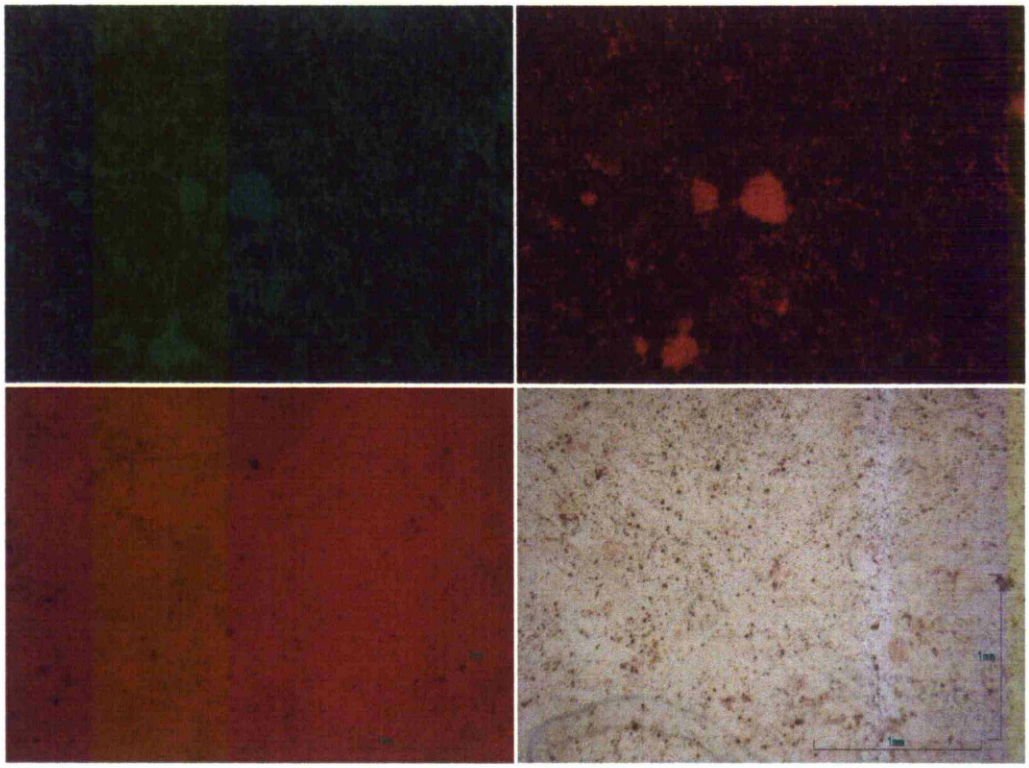

Figure 2.28: IL1β pre-treated osteoblasts cultured for 72 hours in DMEM with addition of 10% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

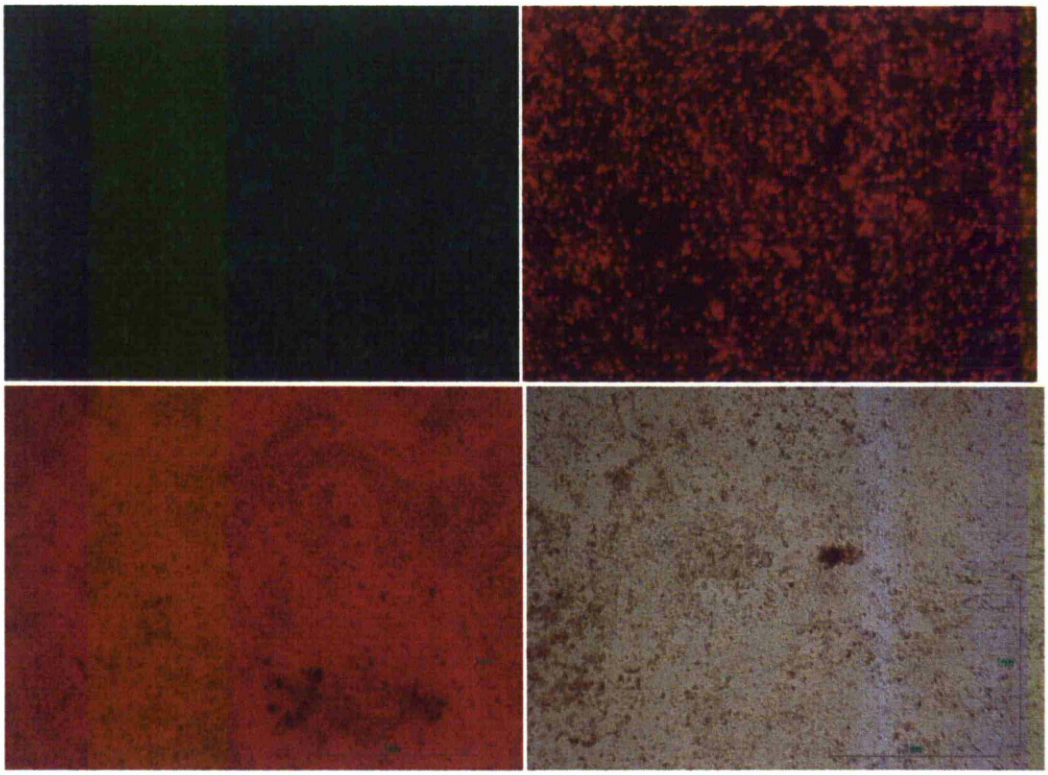

Figure 2.29: Non IL1β pre-treated osteoblasts cultured for 144 hours in serum free DMEM. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

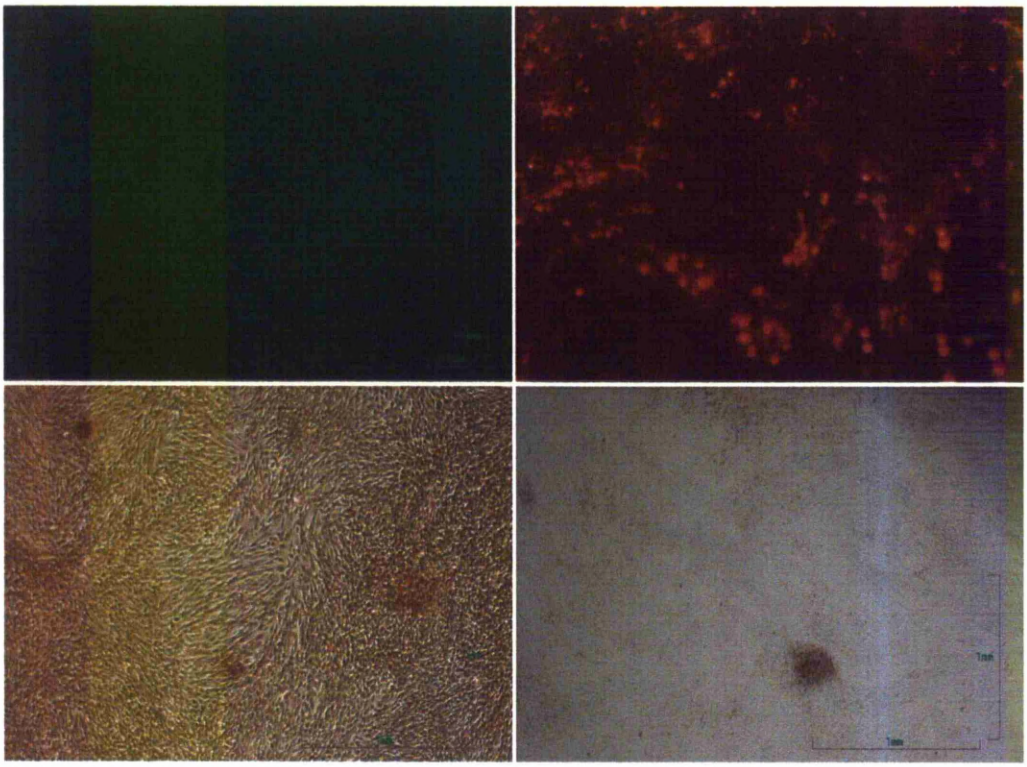

Figure 2.30: IL1β pre-treated osteoblasts cultured for 144 hours in serum free DMEM. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

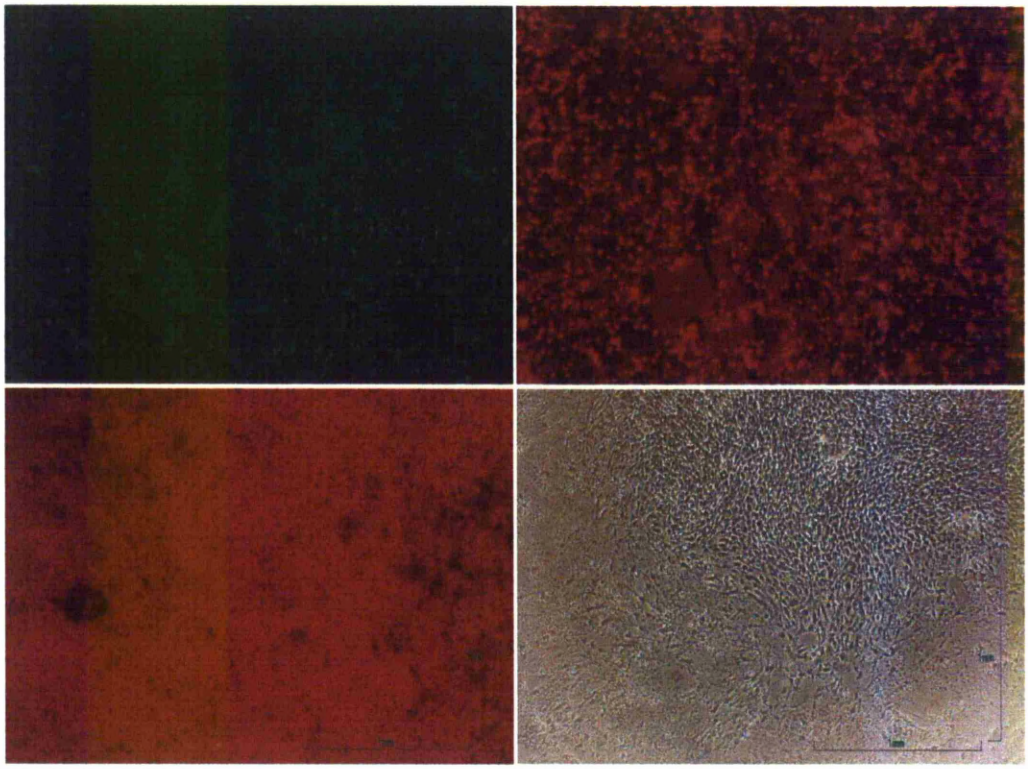

Figure 2.31: Non IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 1% ITS+. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

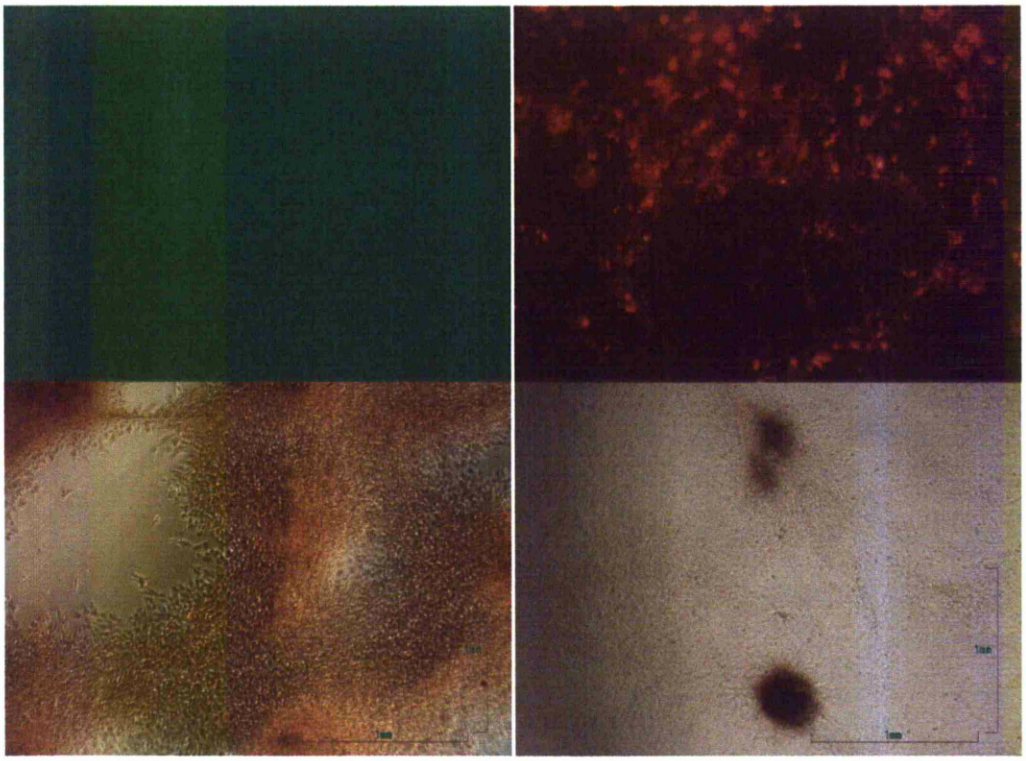

Figure 2.32: IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 1% ITS+. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

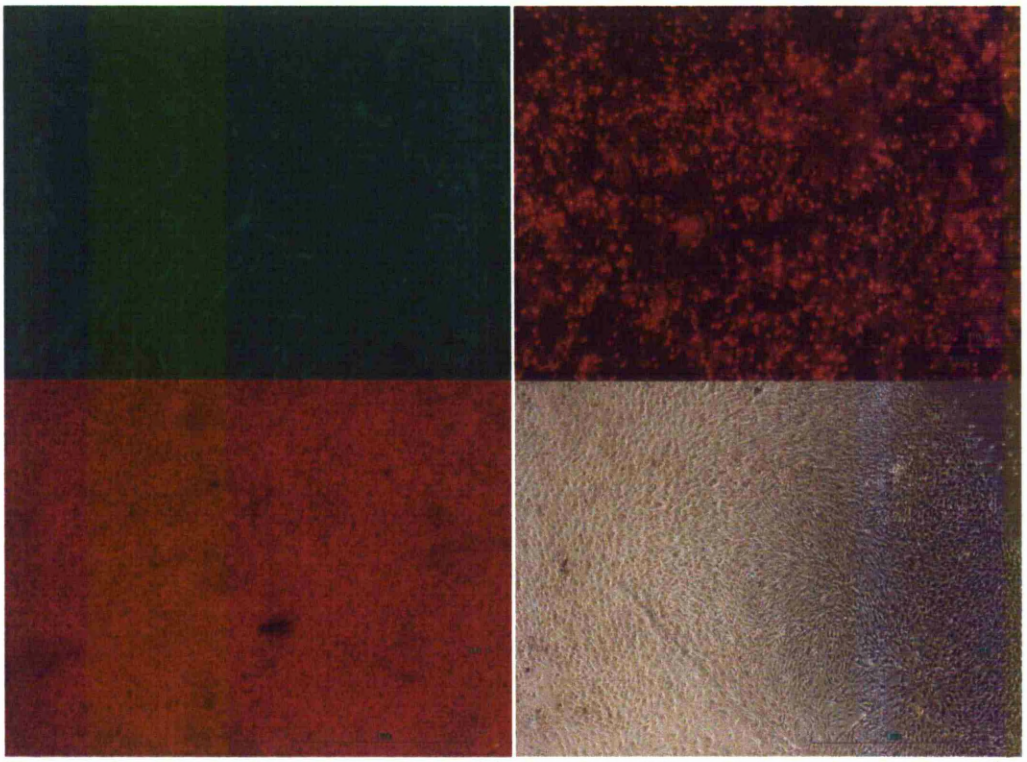

Figure 2.33: Non IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 1% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

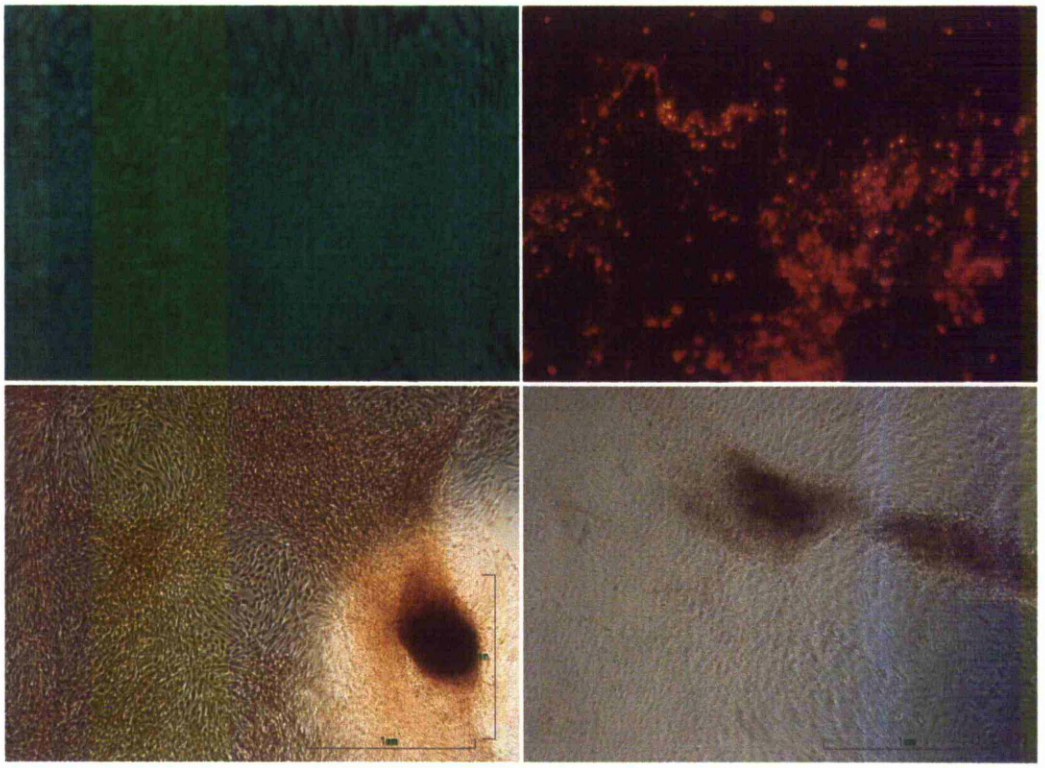

Figure 2.34: IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 1% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

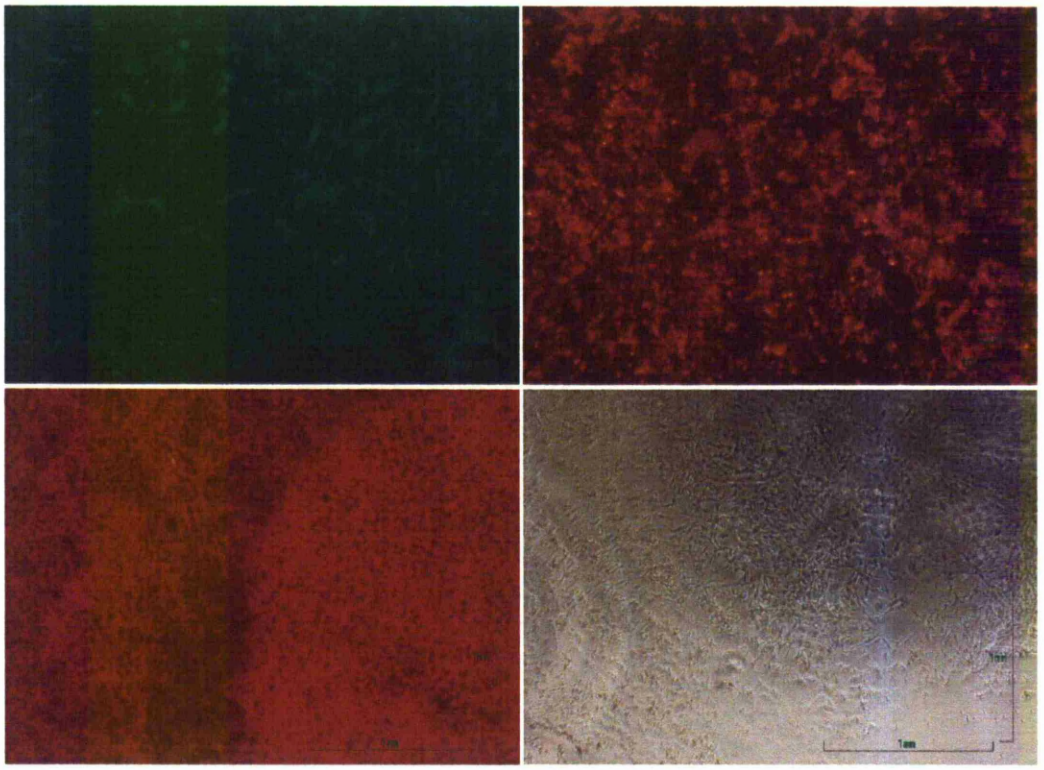

Figure 2.35: Non IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 10% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

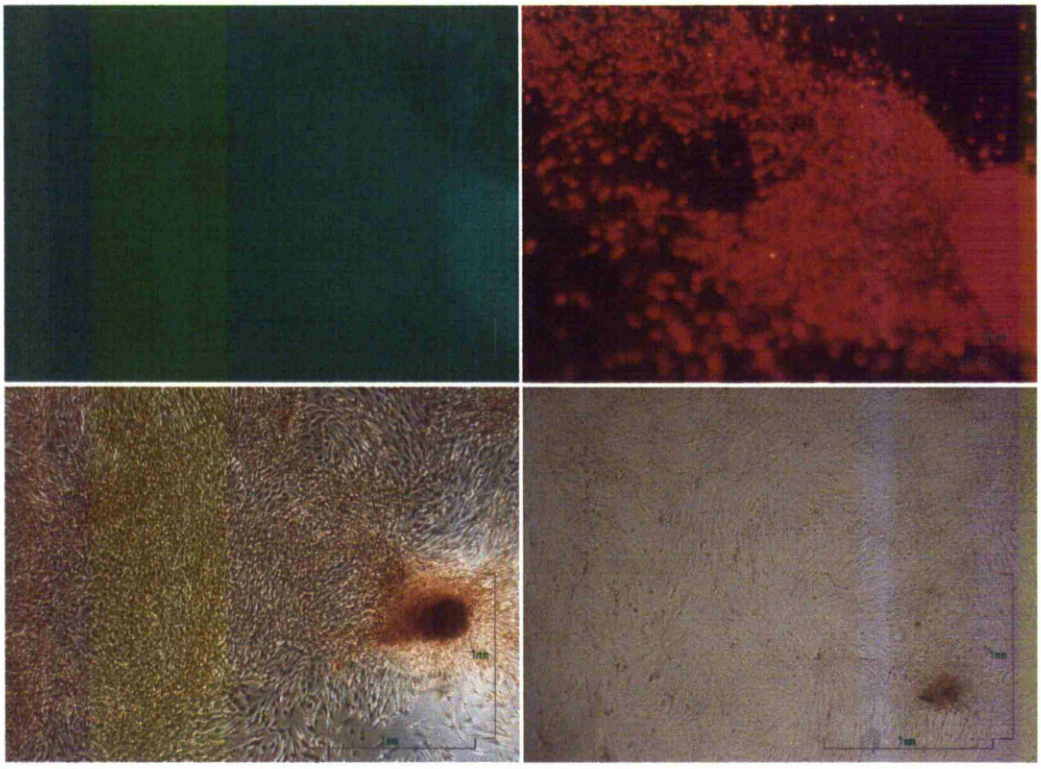

Figure 2.36: IL1β pre-treated osteoblasts cultured for 144 hours in DMEM with addition of 10% FBS. Clockwise from top left: live staining, dead staining, Von Kossa staining, Alizarin Red staining.

2.3.5.1 Seventy two hours culture

Subjectively, at 72 hours culture there was increased dead staining in IL1β pre-treated osteoblasts cultured in serum free DMEM as compared to all other culture conditions. In the live staining, there was evidence of more obvious bone nodule formation and bone autofluorescence in the osteoblasts cultured in DMEM supplemented with 1% ITS+ and 10% FBS as compared to serum free and 1%FBS. Generally, Alizarin Red staining was good for all culture conditions regardless of media used or whether the cells were pre-treated with IL1β. Von Kossa staining was subjectively less marked in both IL1β pre-treated and non pre-treated osteoblasts cultured in serum free DMEM as compared to the other 3 culture media.

2.3.5.2 One hundred and forty four hours culture

Live staining appeared reduced in cells cultured for 144 hours in serum free DMEM. In the other 3 culture media, live/dead staining was similar regardless of whether cells had been pre-treated or not with IL1β. Increased dead staining was observed in all culture media and treatments at 144 hours compared to 72 hours. There was a marked decrease in Alizarin Red staining in the IL1β pre-treated osteoblasts at 144 hours in all culture media as compared to at 72 hours. Although there were isolated nodules of positive Alizarin Red stain in these cells, particularly in the 1% ITS+, 1% FBS and 10% FBS cultures, the characteristic diffuse red staining was lost. Non IL1β pre-treated osteoblasts continued to stain diffusely positively for Alizarin Red in all culture media examined. Von Kossa staining was also reduced in the IL1β pre-treated osteoblasts in all media, and this effect was most marked in the serum free medium.

2.4 Discussion

Bone formation comprises a complex and ordered sequence of events involving the proliferation and differentiation of osteoblastic precursor cells ultimately leading to the formation of a calcified extracellular matrix (Stein *et al.* 1989). Although the process may be observed *in vivo*, it is difficult to study at the molecular level under these conditions, thus various *in vitro* models have been developed to facilitate such studies (Scutt *et al.* 1992). While much is known about these models in the human and laboratory animals, there is very little published data in the equine. Cells grown from explants at first passage are a mixed cell population containing osteoprogenitor cells from the bone matrix, further differentiated osteogenic cells, fibroblasts, chondrogenic cells and pluripotent cells (Ashton *et al.* 1985). This mixed population thus requires further differentiation before it can be effectively used in experiments to investigate the biology of the equine osteoblast.

As a first step in these experiments, the use of two published osteogenic differentiation media were compared to determine which was most appropriate for use with equine cells. There is no one reliable measurement of osteoblast phenotype, therefore a combination of methods is required.

Those utilised in this set of experiments included measurement of osteocalcin expression at gene level, measurement of cellular alkaline phosphatase at protein level, and histochemical staining of the cells and their mineralised matrix.

Osteocalcin has been shown to be an accurate marker of osteoblast phenotype which is expressed during the matrix maturation and mineralisation phases of bone development (Stein *et al.* 1989). Protein levels of osteocalcin were not considered here, but previous authors have shown that gene expression of osteocalcin begins to increase after 7 days of differentiation with a resultant exponential increase in levels of osteocalcin in the culture medium after this point (Sanchez *et al.* 2008).

Alkaline phosphatase is a useful marker of osteoblast phenotype and bone formation, being expressed during the early stages of differentiation and bone matrix deposition, with down-regulation of expression and production during the later stages of matrix mineralisation (Stein *et al.* 1989).

Alizarin Red and von Kossa are histochemical staining methods used to identify mineralisation of the matrix. Both staining methods were used as neither staining method is absolutely reliable in the identification of mineralisation and discrepancies have been found between the two techniques (Puchtler *et al.* 1969). Von Kossa staining has been shown to detect phosphate rather than calcification and the presence of phosphate *per se* does not necessarily imply the presence of calcium or of hydroxyapetite (Bonewald *et al.* 2003). Alizarin Red staining is not specific for calcium with alizarin reacting with other cations and the anion of the salt can affect the colour of the alizarin-metal compound, furthermore the colour of the alizarin-metal compound can alter with pH of the solution (Puchtler *et al.* 1969).

The culture medium containing β-glycerophosphate, L-ascorbic acid and dexamethasone resulted in the most effective differentiation of primary equine osteoblast-like cells. β-glycerophosphate is

utilised as a source of additional phosphate ions after it was shown by Tenenbaum and Heersche (1982) that addition of β-glycerophosphate to the culture media was required for effective mineralisation to occur *in vitro*. The rationale of utilising β-glycerophosphate as the additional source of phosphate ions is that the possibility of nonspecific mineral precipitation is reduced (Tenenbaum and Heersche 1982) since phosphate ions are made available through enzymatic hydrolysis of β-glycerophosphate by alkaline phosphatase produced by osteoblasts. Some authors have questioned the physiological relevance of a cell culture system which requires addition of apparently unphysiological levels of phosphate ions (Beresford *et al.* 1993), however, it has been argued that although inorganic phosphate levels are within the physiologic range (1mM) in culture medium, the organic phosphate levels (4mM) are in fact well below the normal physiologic levels (10mM) and supplementation is a viable means of mimicking the physiological situation (Tenenbaum and Heersche 1982).

L-ascorbic acid is required in cell culture of appropriate cells to stimulate the formation and hydroxylation of collagen, thus allowing the deposition of a collagenous matrix (Barnes 1975). While it has been shown that bone nodules were formed *in vitro* by cells obtained from rat calvaria cultured with L-ascorbic acid, these nodules were unmineralised and it was concluded that addition of both L-ascorbic acid and β-glycerophosphate was required for production of mineralised osteoid nodules in this cell culture system(Bellows *et al.* 1986).

Dexamethasone was added to the culture medium in this series of experiments. It has previously been shown that glucocorticoids at physiological doses enhance osteogenic differentiation by increasing the formation of bone nodules and the expression of genes associated with osteoblast phenotype in primary rat osteoblast cultures (Bellows and Aubin 1989; Bellows *et al.* 1987; Bellows *et al.* 1990; Shalhoub *et al.* 1992). It appears that low dose glucocorticoids promote changes in cell-cell and cell-extracellular matrix signalling such that growth and differentiation of cells capable of osteogenic phenotype development is supported, while growth of cells that cannot progress to the

mature osteoblast phenotype is inhibited (Shalhoub *et al.* 1992). Further, it has been suggested that a potential mechanism for these effects is modulation of expression and action of the bone morphogenic proteins, which are secreted proteins capable of inducing cartilage or bone formation (Cooper *et al.* 1999).

Pre-treatment of bone explants with hyaluronidase and collagenase was performed prior to further culture as described previously (Hilal *et al.* 1998; Sanchez *et al.* 2005). Collagenase pre-treatment was previously described by Robey and Termine (1985) and Robey (1995), who showed that collagenase pre-treatment of bone fragments to be used in explant culture resulted in removal of all cells other than those immediately adjacent to the bone surface. This therefore gives a purer osteoblastic cell population by removing adherent and remaining bone marrow cells (Robey 1995). Whether collagenase treatment of explants is necessary is debatable as three isolation techniques have been compared (Jonsson *et al.* 1999) and it was shown that human cells grown from explants had similar phenotypical characteristics regardless of collagenase treatment. The cells obtained from bone explant culture were shown to differ significantly from those obtained from bone marrow stromal cells by expressing lower levels of alkaline phosphatase, higher levels of type I collagen and higher levels of vitamin D stimulated osteocalcin secretion.

It was demonstrated that pre-treatment of osteoblasts with IL1β resulted in no significant effect on cellular alkaline phosphatase levels. These results support those of Evans et al. (1989) who cultured human osteoblasts with IL1β. In combination with the results of Alizarin Red and Von Kossa staining of cells at 72 hours, it can be proposed that IL1β treatment did not have a detrimental effect on osteoblast differentiation at the end of the culture period. Similarly, there was no reduction in expression of bone specific *COL1A2* or of the proteoglycan biglycan which may indicate that cells maintained an osteoblast phenotype *in vitro* after pre-treatment with IL1β. Interestingly, expression of *COL2A1* was significantly decreased in response to IL1β pre-treatment, which is the same response as has been reported in equine chondrocytes (Richardson and Dodge 2000). This may

indicate that equine osteoblasts in cell culture are responding to IL1β in a similar fashion to other cells of mesenchymal origin. Alternatively, there may be a mixed population of cells such that there are still some undifferentiated fibroblasts in the cell culture. As described above, osteoblast phenotype and differentiation were determined. It would also have been useful to determine fibroblast phenotype, but no marker is available for fibroblast differentiation in the equine.

Increases in expression of mRNA for various matrix metalloproteinases (*MMP1, MMP3* and *MMP13*) were demonstrated after treatment of osteoblasts with IL1β. Although previously the osteoclast was considered to be the main producer of proteinases in bone, there is now mounting evidence of the importance of the role of the osteoblast in production of these enzymes. The osteoblast has the capacity therefore to synthesise a range of matrix proteins and has the ability to remodel its own extracellular matrix through the secretion of a range of proteinases (Bilezikian *et al.* 2008). In an experiment with time points looking at *MMP3* expression, it was shown that gene expression was time-dependent, being highest 24 hours after IL1β removal, decreasing at 48 hours and decreasing again at 72 hours. However, gene expression remained significantly higher at 72 hours after IL1β removal as compared to basal levels in untreated osteoblasts. In a subsequent experiment it was demonstrated that significant increases in gene expression were evident for all MMPs measured (*MMP1, MMP3* and *MMP13*) at 72 hours of further culture after the IL1β was removed. How this corresponds with the levels of protein and of active and inactive enzyme was not investigated in these experiments, although this would have been a useful adjunct and has been addressed using MMP13 assays of the media in co-culture experiments in Chapter 3. Previous authors have measured secretion of MMP1 and MMP3 and failed to show secretion of these proteins in the medium of normal human osteoblasts cultured with IL1β (Rifas *et al.* 1994).

No alteration in *ADAMTS 4* or *ADAMTS5* expression in response to IL1β were observed in this model despite the previous implication of ADAMTS5 in subchondral bone disease and osteoarthritis (Botter

et al. 2009). *ADAMTS5* gene expression has previously been shown to increase in response to IL1β in bovine chondrocytes (Chan *et al.* 2006; Cortial *et al.* 2006) and *ADAMTS4* gene expression has been shown to increase in response to IL1β in human chondrocytes (Bau *et al.* 2002; Moulharat *et al.* 2004), bovine chondrocytes (Cortial *et al.* 2006) and human synovial fibroblasts . While it has been shown that the osteoblast expresses *ADAMTS4* and *ADAMTS5* in rat bone (Nakamura *et al.* 2005), there is no published data in any species on alterations in expression of these genes in response to IL1β. Therefore the failure to show any alterations in expression of *ADAMTS4* or *ADAMTS5* in equine osteoblasts in response to IL1β may be as a result of species differences, or because *ADAMTS4* and *ADAMTS5* expression in the osteoblast are indeed not mediated via IL1β.

Pre-treatment of osteoblasts with 10ng/ml IL1β for 24 hours prior to a further period of 72 hours of culture in non-osteogenic media does not appear to have a detrimental effect on osteoblast differentiation. Alterations in gene expression were evident in response to application of IL1β and remained for 72 hours after removal of IL1β. When culture times after removal of IL1β were extended to 144 hours, osteoblast survival and differentiation appeared to benefit from addition of 1% ITS+, 1% FCS or 10% FCS to the culture medium. At 144 hours of culture after removal of IL1β in those osteoblasts that were pre-treated, there was evidence of decreased mineralisation of the matrix characterised by reduced von Kossa and Alizarin Red staining. As this was not seen in the osteoblasts not pre-treated with IL1β at 144 hours or in either the IL1β pre-treated or not pre-treated group at 72 hours, it is difficult to explain how the osteoblasts can lay down a mineralised matrix, only for this to disappear after a further period of culture. The use of live/dead staining gave some indication as to the survival cells, however this technique was limited in this system by the lack of quantitative data and by the autofluorescence of bone/mineralised matrix.

2.5 Conclusion

It has been shown that osteoblasts can be grown from equine subchondral osteoblasts and have an ability to differentiate and lay down a mineralised matrix when cultured in the presence of an osteogenic culture media containing β-glycerophosphate, dexamethasone and L-ascorbic acid at physiological levels. These cells respond to IL1β and this model may be used for further investigation of the cell biology of subchondral bone disease in osteoarthritis.

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Co-Culture System of Equine Osteoblasts and Chondrocytes

3.1 Introduction

Clinically, the role of subchondral bone in the pathogenesis of osteoarthritis (OA) is well recognised and *in vitro* there is mounting evidence of an association between the altered phenotypes of the chondrocytes and osteoblasts in the development of disease. Westacott et al. (1997) showed that OA osteoblasts grown in monolayer increased glycosaminoglycan release from cartilage explants, whereas normal cells did not. Sanchez et al. (2005a, b) showed that osteoblasts from sclerotic areas of osteoarthritic subchondral bone induced phenotypic changes in co-culture with osteoarthritic chondrocytes and that certain of these phenotypic changes could be mimicked by treating osteoblasts obtained from areas of non-sclerotic bone from joints affected with OA with interleukin-6, interleukin 1β and oncostatin M.

It is generally understood that cytokines such as interleukin 1β (IL1β) are involved in the pathways leading to osteoarthritis. The effect of IL1β on equine chondrocytes has previously been investigated (David *et al.* 2007; May *et al.* 1992; Richardson and Dodge 2000). The effect of co-culture of equine cartilage explants with synoviocytes has also been described (Gregg et al. 2006). However, to our knowledge, there have been no previous reports of co-culture of equine cartilage explants with osteoblasts. The advantage of using equine tissue is that normal subchondral bone and cartilage tissue from individuals not affected by osteoarthritis can be obtained more readily, unlike studies using human tissue where material largely comes from patients undergoing joint replacement where osteoarthritis is already at end stage.

The aim of this set of experiments was to develop a co-culture system of equine osteoblasts and cartilage explants. It had been previously shown (Chapter 2) that the phenotype of equine differentiated osteoblasts was altered in response to stimulation with IL1β, with increases in expression of genes involved in matrix proteolysis. It was hypothesised that co-culture of equine

osteoblasts pre-treated with IL1β with cartilage explants from joints unaffected by OA would result in an altered chondrocyte phenotype and evidence of cartilage matrix degradation.

3.2 Materials and Methods

3.2.1 Tissue Collection

Material was collected either from horses euthanased at The University of Liverpool Equine Hospital for reasons unrelated to orthopaedic disease/osteoarthritis and where owners had given informed consent for use of samples from their horse, or from healthy horses euthanased at a local abattoir. All material was gathered within 12 hours of the horse's death. Although exact ages of abattoir cases were not available, all subjects were mature and all joints from which samples were obtained were grossly free of osteoarthritis. Cartilage and subchondral bone were obtained from either the metacarpo- or metatarsophalangeal joint. Full-thickness cartilage from was obtained from the dorsal and palmar/plantar aspects of both lateral and medial condyles by sterile dissection with a scalpel blade. The subchondral bone plate was removed from the palmar/plantar aspect of both lateral and medial distal condyles by sterile dissection with a chisel, such that small fragments of bone with dimensions approximately 3mm x 3mm were obtained.

3.2.2 Subchondral Osteoblasts in Monolayer Culture

Unless stated otherwise, all reagents were supplied by Sigma-Aldrich (Dorset, UK). Based on the protocols of Hilal et al. (1998) and Sanchez et al. (2005) the small pieces of subchondral bone were sequentially incubated in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) at 37°C, initially with 0.5mg/ml hyaluronidase type IV S for 20 minutes then with 0.6mg/ml collagenase IA for 4 hours. The digested bone pieces were then washed in Hank's Balanced Salt Solution (HBSS) before culturing in T-75 flasks in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 500ng/ml amphotericin B. Medium was changed twice weekly until cells were observed to have migrated from the bone explants and had reached

confluence. At this point cells were collected by trypsinisation, seeded at 20,000 cells/cm^2 in 12 well plates and grown for 12 days in an osteogenic differentiation medium, composed of DMEM supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B, 10mM β glycerophoshate, 0.1mM L-ascorbic acid-2-phoshate and 10nM dexamethasone. At the end of this differentiation period, cells were shown to have an osteoblastic phenotype characterised by the production of alkaline phosphatase, gene expression of osteocalcin and positive staining with Alizarin Red and von Kossa stains. At the end of the differentiation period and after washings in HBSS, half of the plates of osteoblasts were treated with 10ng/ml IL1β in DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+ for a period of 24 hours. The remainder of the plates of osteoblasts were washed in HBSS and cultured in DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+ for the same 24 hour period. Prior to setting up the co-culture experiments, all osteoblasts were washed 3 times in HBSS.

3.2.3 Cartilage Explants

Cartilage was obtained on the same day as IL1β stimulation of the osteoblasts. Explants were cut into small pieces (5mm x 5mm approx) and left overnight at 37°C in DMEM supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 500ng/ml amphotericin B. Cartilage explants were then washed and utilised in the co-culture system.

3.2.4 Osteoblasts/Cartilage Explant Co-Culture

Co-cultures were set up with allogeneic cartilage explants (1 per co-culture) being placed in a co-culture insert with a pore size of 1µm (Greiner-Bio, Stonehouse, UK) and co-cultured with osteoblasts. Experimental design of cultures was as follows:

1) Osteoblasts previously incubated with IL1β co-cultured with cartilage explants (O+IL1+C)

2) Osteoblasts not previously incubated with IL1β co-cultured with cartilage explants (O-IL1+C)
3) Osteoblasts previously incubated with IL1β (O+IL1-C)
4) Osteoblasts not previously incubated with IL1β (O-IL1-C)
5) Cartilage explants cultured alone without osteoblasts (C-O)
6) Cartilage explants cultured with IL1β (C+IL1)

Cultures were carried out for 72 hours (gene expression, MMP 13 assays, alkaline phosphatase assays) or 6 days with media collection every 48 hours (GAG assays). The media used for the 72 hour co-cultures was DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+ and that for the GAG assays was DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B and 1% FBS (see Appendix A). For each part of the experiment, cultures were performed in triplicate. In total co-cultures were performed 4 times, with subchondral bone and cartilage being retrieved from different donors in each co-culture such that a total of 8 donors were used.

3.2.5 Alizarin Red and Von Kossa's Staining

Alizarin Red and von Kossa's Staining were performed to ascertain differentiation of the osteoblasts and confirm osteoblast phenotype. Media was removed from the wells and cells were washed twice in phosphate buffered saline (PBS) prior to fixing of the cells in 4% formaldehyde for 10 minutes. Cells were washed twice in ultra-pure water before application of the stain. For the Alizarin Red stain, 1% Alizarin Red was applied for 5 minutes then washed twice in ultra pure water. For the von Kossa stain, 1% silver nitrate was applied to the cells and then placed under ultraviolet light for 40 minutes. Cells were washed in distilled water then treated with 3% sodium thiosulphate for 5 minutes.

3.2.6 Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was quantified in the cellular fraction of the osteoblast culture. Media was removed at the end of the co-culture period (72h). Cells were washed in PBS then lysed in 1ml 0.2% Triton X-100 and the Triton-X/cell lysate mixture was collected. Cell extract (5µL) was incubated with 195µL of a 10mM p-Nitrophenyl phosphate working solution (BioAssay Systems, Hayward, USA). A standard solution of tartrazine was used as a calibrator, according to the kit's instructions. In the presence of alkaline phosphatase, p-Nitrophenyl phosphate is converted to p-nitrophenol and inorganic phosphate, with the rate of the reaction being directly proportional to the enzyme activity. The absorbance of p-nitrophenol was measured at 405nm on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland)at room temperature at t=0 and t=4 minutes. ALP activity of the sample is measured according to the kit's instructions as:

$$\text{ALP (IU/L)} = \frac{(\text{OD sample t} - \text{OD sample 0}) \times \text{Reaction Volume}}{(\text{OD calibrator} - \text{OD } H_2O) \times \text{Sample Volume} \times t} \times 40.4$$

Alkaline phosphatase activity was normalised to total protein content of the cellular fraction.

3.2.7 Total Protein Assay

Total protein content of the osteoblast cellular fraction collected in 0.2% Triton X-100 was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Thermo Scientific, Rockford, Illinois) according to the manufacturer's instructions. This technique is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. Cu^{1+} is detected in the presence of bicinchoninic acid (BCA) by the chelation of 2 molecules of BCA with one Cu^{1+} ion resulting in a purple-coloured reaction product. The assay was measured on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland) at 570nm relative to standards of albumin with known protein concentration.

3.2.8 Quantitative Real-Time PCR

3.2.8.1 RNA extraction and Reverse Transcription

Co-cultures were terminated at 72 hours. Osteoblasts were harvested in TRI® Reagent. Cartilage explants were stored in RNA later (Ambion, Applied Biosystems, Warrington, UK), and subsequently dismembranated then stored in TRI® Reagent. RNA extraction was performed using a standard chloroform and ethanol extraction followed by the RNeasy (Qiagen, Crawley, UK) column technique, incorporating a DNase treatment stage. RNA was quantified using a Nanodrop (Thermo Scientific, Wilmington, USA) and 1μg of RNA or the maximum volume for the reaction (12.4μL) where RNA concentration was < 80.6ng/μL, was used as the template for the reverse transcriptase reaction. cDNA strands were generated from the RNA in a 25μL volume reaction using Random Primer (Promega, Southampton, UK), RT buffer, 10mM of dNTPs, M-MLV RT enzyme and RNase inhibitor.

3.2.8.2 Primer Preparation

Equine specific PCR primers were designed by obtaining equine RNA and DNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/). Where the equine sequence was unavailable, a multiple species alignment was performed (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and primers designed where there was sequence homology across species. Exon boundaries were identified (http://www.ensembl.org/index.html) and where possible primers were designed to amplify across exon-intron boundaries to allow discrimination of genomic DNA and cDNA. As this was not possible in all cases, the DNase treatment stage was incorporated in the RNA extraction technique as above. Primers were designed based on the sequences acquired using Primer Express software (Applied Biosystems) or Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer efficiencies were validated using a standard curve derived from equine cDNA using a 10-fold dilution series with 5 measuring points. The primers used for the target and housekeeping genes are as shown in Table 3.1.

Gene	Primer Sequence	Accession Code	Efficiency
GAPDH	F: GCATCGTGGAGGGACTCA R: GCCACATCTTCCCAGAGG	AF157626	-3.32
ADAMTS4	F: CAGCCTGGCTCCTTCAAAAA R: CCGCAGGAATAGTGACCACAT	NM_001111299	-3.23
ADAMTS5	F: ACCGATCCTGCAGTGTCACA R: CTGCTCATGGCGAAAAGATTT	EU025851	-3.13
MMP1	F: GGTGAAGGAAGGTCAAGTTCTGAT R: AGTCTTCTACTTTGGAAAAGAGCTTCTCT	NM_001081847	-3.36
MMP3	F: TCTTGCCGGTCAGCTTCATATAT R: CCTATGGAAGGTGACTCCATGTG	NM_001082495	-3.63
MMP13	F: CTGGAGCTGGGCACCTACTG R: ATTTGCCTGAGTCATTATGAACAAGAT	NM_001081804	-3.51
TIMP3	F: CTGCAACTTCGTGGAGAGGT R: ACTCGTTCTTGGAGGTCACG	NM_001081870	-3.54
Collagen I α2	F: GCACATGCCGTGACTTGAGA R: CATCCATAGTGCATCCTTGATTAGG	XM_001492939	-3.31
Collagen II α1	F: TCAAGTCCCTCAACAACCAGATC R: GTCAATCCAGTAGTCTCCGCTCTT	NM_001081764	-3.21
Biglycan	F: TCACCTTCCAGCCCCTAGAGT R: AGAAGCAGCCCCTCCTCAA	NM_001081839	-3.71
Aggrecan	F: GAGGAGCAGGAGTTTGTCAACA R: CCCTTCGATGGTCCTGTCAT	XM_001499504	-3.81
Osteocalcin	F: TCAACCCAGACTGTGACGAG R: CAGCTAGGGACGATGAGGAC	XM_001915727	-3.80

Table 3.1: Primer sequences used for quantitative real-time PCR
F= Forward primer, R= Reverse primer

3.2.8.3 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was performed in a 25µL volume using SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) and 300nM primer concentration and processed by 7300 Real Time PCR system (Applied Biosystems, Warrington, UK) using standard amplification conditions. Data was analysed using SDS software (Applied Biosystems, Warrington, UK). PCR products were measured and normalised against GAPDH as a housekeeping gene.

3.2.9 Fluorometric Activity Assay for MMP-13

The enzymatic activity of MMP-13 in the culture medium was assayed using a fluorogenic substrate (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2;Merck, Nottingham, UK) that specifically detects MMP-13. Forty µL of culture media was mixed with 50µL buffer (50mM HEPES, 200mM NaCl, 1mM $CaCl_2$ and 0.01% v/v Brij-35 at pH 7.3) or 50µL 1mM p-aminophenylmercuric acetate (APMA) in assay buffer (as

above) to give a final concentration of 0.5mM of APMA per well. APMA acts to activate the latent or pro-MMPs and therefore gives a measurement of total MMP. Assays performed without the addition of APMA gave a measurement of the total active MMP13 in the sample. Assays were incubated at 37°C for 1 hour to activate the latent enzyme in those samples in which APMA had been added, then fluorogenic substrate was added at a final concentration of 1μM per well. Substrate hydrolysis was measured on a FL_X800 microplate fluoresecence reader (Winooski, Vermont, USA) with excitation and emission wavelengths set at 325 and 393nm respectively. A linear standard curve with dilutions of 1:2, 1:4, 1:8, 1:16 and media control was performed to ensure that readings were linear and within the required range. Final readings were taken at t=120 minutes.

3.2.10 Glycosaminoglycan Spectrophotometric Analysis

Harvested cartilage explants were incubated overnight at 60°C in 500μL of papain (10units/ml) in digest buffer (0.1M sodium acetate, 2.4mM EDTA disodium salt and 5mM L-cysteine at pH 5.8). Aliquots of media (40μL) and of digested explants (2μL diluted with 38μL H_2O) were analysed for glycosaminoglycan (GAG) content using the 1.9-dimethylene blue (DMMB) labelling technique as described by Farndale et al. (1986). Briefly, aliquoted media and explant papain digests were incubated with 250μL DMMB (16μg/ml 1-9 dimethyl methylene blue, 2mg/ml sodium formate and 0.2% v/v formic acid at pH 3.5). Sulphated GAG concentrations were determined by absorbance readings on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland) at 570nm relative to a shark chondroitin sulphate C standard curve (0 to 70μg/ml). Cumulative GAG release data was expressed as both a percentage of the total available GAG released into the media and as GAG loss into the media per μg DNA of the papain digest.

3.2.11 DNA Assay

The DNA contents of papain digests of explants were determined using a Quanti-iT™ PicoGreen® dsDNA Assay (Invitrogen, Paisley, UK) measured on a FL_X800 microplate fluoresecence reader (Winooski, Vermont, USA) at 480nm excitation and 520nm emission.

3.2.12 Statistical Analysis

After plotting and testing for normality using the Kolmogrov-Smirnov test, data was logged where required using Mintab (v.15; Minitab Inc., Pennsylvania). Mixed effects linear regression (SPlus v. 6.1; TIBCO Software Inc., California) was used to test for significant differences between groups whilst allowing for the clustering of samples within donor. Significance was set at $P<0.05$ unless multiple comparisons were made on the same sample (i.e. gene expression data), where the P-value was adjusted using Sidak's formula (Sidak 1967):

$$\alpha_{PT} = 1 - (1-\alpha_{PF})^{1/c}$$

where α is the probability of making a Type I error, α_{PT} is the α per test, α_{PF} is the α per family of tests and c is the number of comparisons. Therefore if we assume that the required α_{PF} is 0.05, then:

$$\alpha_{PT} = 1 - (1-0.05)^{1/c}$$

3.3 Results

3.3.1 Alizarin Red and Von Kossa Staining

Osteoblasts were found to stain positively with Alizarin Red and von Kossa stains at the end of the co-culture period.

3.3.2 Alkaline Phosphatase Assay

Levels of alkaline phosphatase were normalised to the total protein content of the cellular fraction in cultures containing osteoblasts and were expressed as IU/L/mg total protein. Levels of alkaline

phosphatase are as shown in Figure 3.1. There was no statistically significant difference (P>0.05) between alkaline phosphatase levels in the cellular fraction of the osteoblasts regardless of the culture condition (i.e. pre-treated or not with IL1β, or co-cultured or not with cartilage explants).

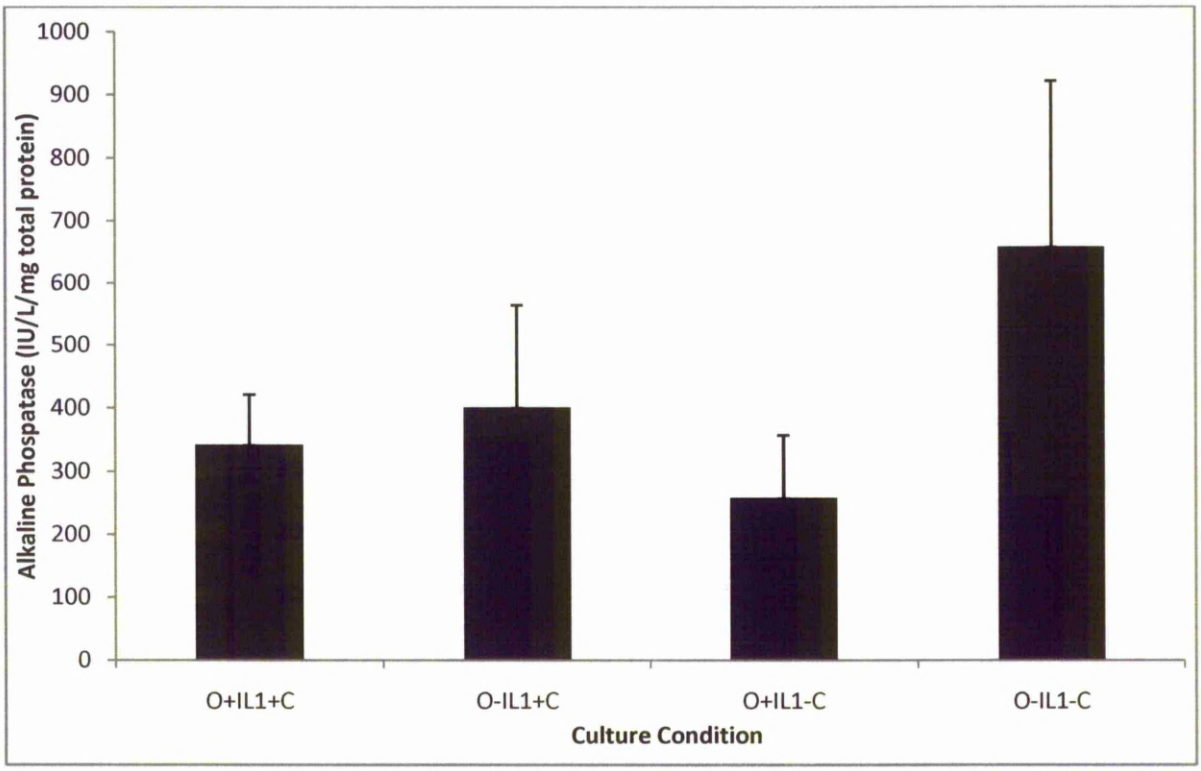


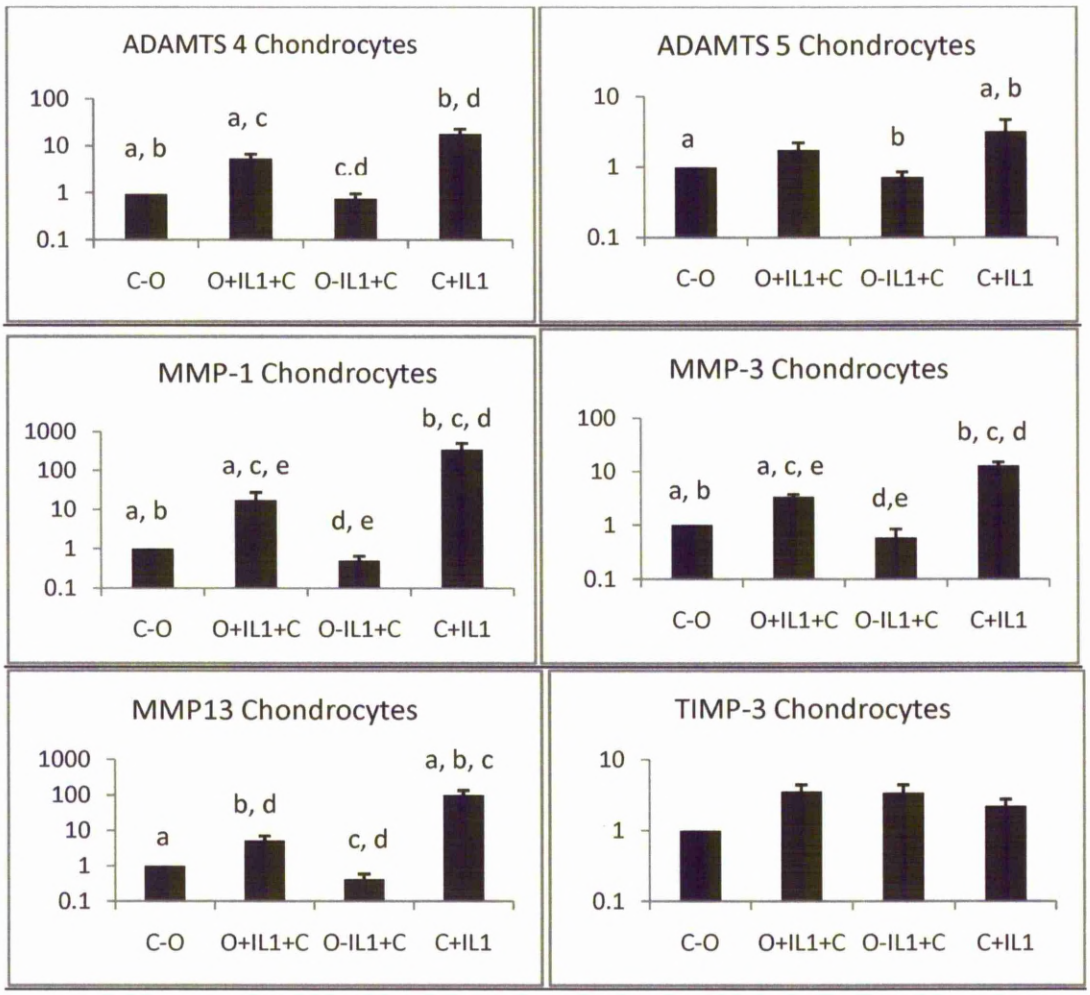
Figure 3.1: Alkaline phoshatase content of the osteoblast cellular fraction normalised to the total protein content of the sample and expressed as IU/L/mg total protein. Error bars indicate standard error. O+IL1+C = osteoblasts pre-treated with interleukin 1β and co-cultured with cartilage; O-IL1+C = osteoblasts not pre-treated with interleukin 1β and co-cultured with cartilage; O+IL1-C = osteoblasts pre-treated with interleukin 1β; O-IL1-C = osteoblasts

3.3.3 Quantitative Real-Time PCR

3.3.3.1 Chondrocytes

Expression of various markers of cartilage degradation: matrix metalloproteinase-1 (*MMP1*), -3 (*MMP3*), -13 (*MMP13*); a disintegrin and metalloproteinase with thrombospondin motifs -4 (*ADAMTS4*), -5(*ADAMTS5*) and tissue inhibitor of metalloproteinase-3 (*TIMP3*) and various markers of matrix synthesis: type I collagen α2 chain (*COL1A2*); type II collagen α1 chain (*COL2A1*); aggrecan (*ACAN*) and biglycan (*BGN*) were measured relative to GAPDH in chondrocytes obtained from

cartilage explants. Differences in gene expression are shown for the chondrocytes as fold differences compared to the negative control i.e. cartilage explants cultured without osteoblasts (C-O). Alterations in gene expression of chondrocytes are as shown in Figures 3.2a) – j). Statistical significance between differences in gene expression for all genes in all culture conditions are as detailed in Table 3.2. Ten genes were tested on each sample, giving a Sidak corrected significance level of P≤0.005.



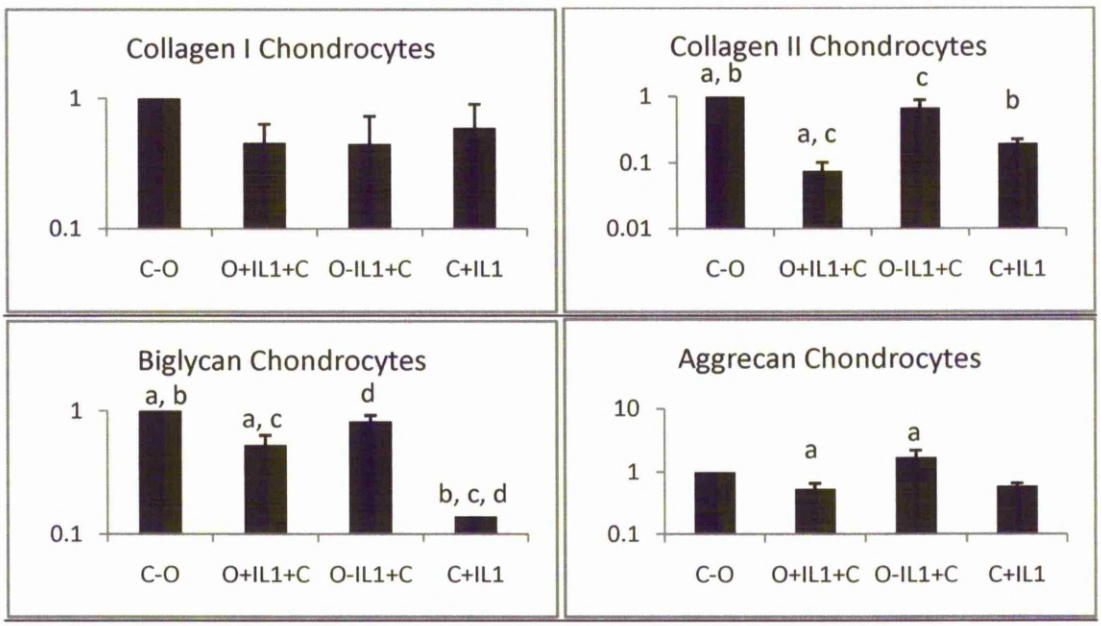


Figure 3.2a-j: Gene expression relative to GAPDH of chondrocytes shown as the log of fold differences compared to cartilage explants cultured alone (C-O). Error bars indicate standard error. C-O= cartilage; O+IL1+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; C+IL1 = cartilage treated with interleukin 1β. Groups with significant differences in gene expression are shown with letters on the graphs and corresponding P values are shown in Table 3.2.

3.3.3.1.1 Effect on chondrocytes of direct application of IL1β to cartilage explants

IL1β was applied directly as to cartilage explants (C+ IL1β) as a positive control and results are included in Figure 3.2 and Table 3.2 as above. In the C+IL1β positive control, compared to the negative control consisting of cartilage explants (C-O), there was; increased expression of *MMP1* (P<0.0001), *MMP3* (P<0.0001), *MMP13* (P<0.0001), *ADAMTS4* (P<0.0001) and *ADAMTS5* (P=0.002); decreased expression of *BGN* (P<0.0001)and *COL2A1* (P=0.0007); and no significant difference in expression of *TIMP3, COL1A2* or *ACAN*.

3.3.3.1.2 Effect on chondrocytes of co-culturing cartilage explants with IL1β pre-treated osteoblasts

Comparing O+IL1+C chondrocytes to C-O chondrocytes there was; significantly increased expression of *MMP1* (P=0.001), *MMP3* (P=0.005) and *ADAMTS4* (P<0.0001); decreased expression of *BGN* (P=0.001) and *COL2A1* (P=0.0003); and no significant difference in expression of *MMP13, ADAMTS5,*

TIMP3, *COL1A2* or *ACAN*. There was a trend toward increased *MMP13* expression, but this just failed to reach statistical significance (P=0.006).

3.3.3.1.3 Effect on chondrocytes of co-culturing cartilage explants with non IL1β pre-treated osteoblasts

Comparing O-IL1+C chondrocytes to C-O chondrocytes there was; no significant difference in expression in any of the genes examined (*ADAMTS4*, *ADAMTS5*, *MMP1*, *MMP3*, *MMP13*, *TIMP3*, *COL1A2*, *COL2A1*, *BGN* and *ACAN*).

Gene	Cell	Culture Condition, Significance of Differences in Gene Expression between Groups and Increase(↑)or Decrease (↓) in Expression (row compared to column)			
ADAMTS4	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P<0.0001 ↑	P=0.01	P=0.0005 ↑
		O-IL1β+C	P=0.3	P<0.0001 ↓	
		C+IL1β	P<0.0001 ↑		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.9	P=0.3	P=0.8
		O-IL1β+C	P=1	P=0.2	
		O+IL1β-C	P=0.3		
ADAMTS5	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.08	P=0.2	P=0.05
		O-IL1β+C	P=0.2	P=0.006 ↓	
		C+IL1β	P=0.002 ↑		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=1	P=0.2	P=0.2
		O-IL1β+C	P=0.2	P=0.03	
		O+IL1β-C	P=0.2		
MMP1	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.001 ↑	P=0.0002↓	P<0.0001↑
		O-IL1β+C	P=0.1	P<0.0001↓	
		C+IL1β	P<0.0001 ↑		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.0001 ↑	P=0.5	P=0.0001 ↑
		O-IL1β+C	P=0.9	P=0.0001 ↓	
		O+IL1β-C	P=0.0001 ↑		
MMP3	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.005 ↑	P=0.003↓	P=0.0002 ↑
		O-IL1β+C	P=0.03	P<0.0001↓	
		C+IL1β	P<0.0001 ↑		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.02	P=0.15	P=0.009
		O-IL1β+C	P=0.6	P=0.002 ↓	
		O+IL1β-C	P=0.003 ↑		
MMP13	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.006	P=0.0001↓	P=0.0002 ↑
		O-IL1β+C	P=0.03	P<0.0001↓	
		C+IL1β	P<0.0001 ↑		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.06	P=0.2	P=0.02
		O-IL1β+C	P=0.3	P=0.003 ↓	
		O+IL1β-C	P=0.001 ↑		
TIMP3	Chondrocyte		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.02	P=0.2	P=0.9
		O-IL1β+C	P=0.03	P=0.2	
		C+IL1β	P=0.2		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C

		O+IL1β+C	P=0.6	P=0.9	P=0.3
		O-IL1β+C	P=0.5	P=0.3	
		O+IL1β-C	P=0.7		
Collagen I	**Chondrocyte**		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.07	P=0.6	P=1
		O-IL1β+C	P=0.07	P=0.6	
		C+IL1β	P=0.2		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.008	P=0.06	P=0.5
		O-IL1β+C	P=0.02	P=0.1	
		O+IL1β-C	P=0.2		
Collagen II	**Chondrocyte**		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.0003 ↓	P=0.5	P=0.005↓
		O-IL1β+C	P=0.07	P=0.02	
		C+IL1β	P=0.0007 ↓		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.0004 ↓	P=0.2	P=0.0004 ↓
		O-IL1β+C	P=0.9	P=0.0001 ↑	
		O+IL1β-C	P=0.0002 ↓		
Biglycan	**Chondrocyte**		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.001 ↓	P=0.004 ↑	P=0.02
		O-IL1β+C	P=0.1	P=0.0001↑	
		C+IL1β	P<0.0001 ↓		
	Osteoblast		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.002 ↓	P=0.04	P=0.3
		O-IL1β+C	P=0.005 ↓	P=0.2	
		O+IL1β-C	P=0.03		
Aggrecan	**Chondrocyte**		C-O	C+IL1β	O-IL1β+C
		O+IL1β+C	P=0.03	P=0.5	P=0.004↓
		O-IL1β+C	P=0.2	P=0.01	
		C+IL1β	P=0.09		
Osteocalcin	**Osteoblast**		O-IL1β-C	O+IL1β-C	O-IL1β+C
		O+IL1β+C	P=0.005 ↓	P=0.2	P=0.04
		O-IL1β+C	P=0.2	P=0.3	
		O+IL1β-C	P=0.03		

Table 3.2: Statistical significance of differences between gene expression of various genes relative to GAPDH in chondrocytes and osteoblasts in different culture conditions. Calculated using mixed effects linear regression, significance level P≤0.005.
C-O= cartilage; O+IL1β+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1β+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; C+IL1β = cartilage treated with interleukin 1β; O-IL1β-C = osteoblasts; O+IL1β-C = osteoblasts pre-treated with interleukin 1β.

3.3.3.2 Osteoblasts

Expression of *MMP1*, *MMP3*, *MMP13*, *ADAMTS4*, *ADAMTS5*, *TIMP3*, *COL1A2*, *COL2A1*, *BGN* and osteocalcin (*BGLAP*) were measured relative to GAPDH in osteoblasts. Differences in gene expression are shown for the osteoblasts as fold differences compared to the negative control i.e. osteoblasts grown in monolayer without cartilage explants or pre-treatment with IL1β (O-IL1β-C). Alterations in gene expression of chondrocytes are as shown in Figures 3.3 a) – j). Statistical significance between differences in gene expression for all genes in all culture conditions are as detailed in Table 3.2. Ten genes were tested on each sample, giving a Sidak corrected significance level of $P \leq 0.005$.

All data is shown, but as alterations in gene expression for osteoblasts in monolayer pre-treated with IL1β have been shown and discussed in depth in a previous chapter, only the co-culture results will be considered in detail here.

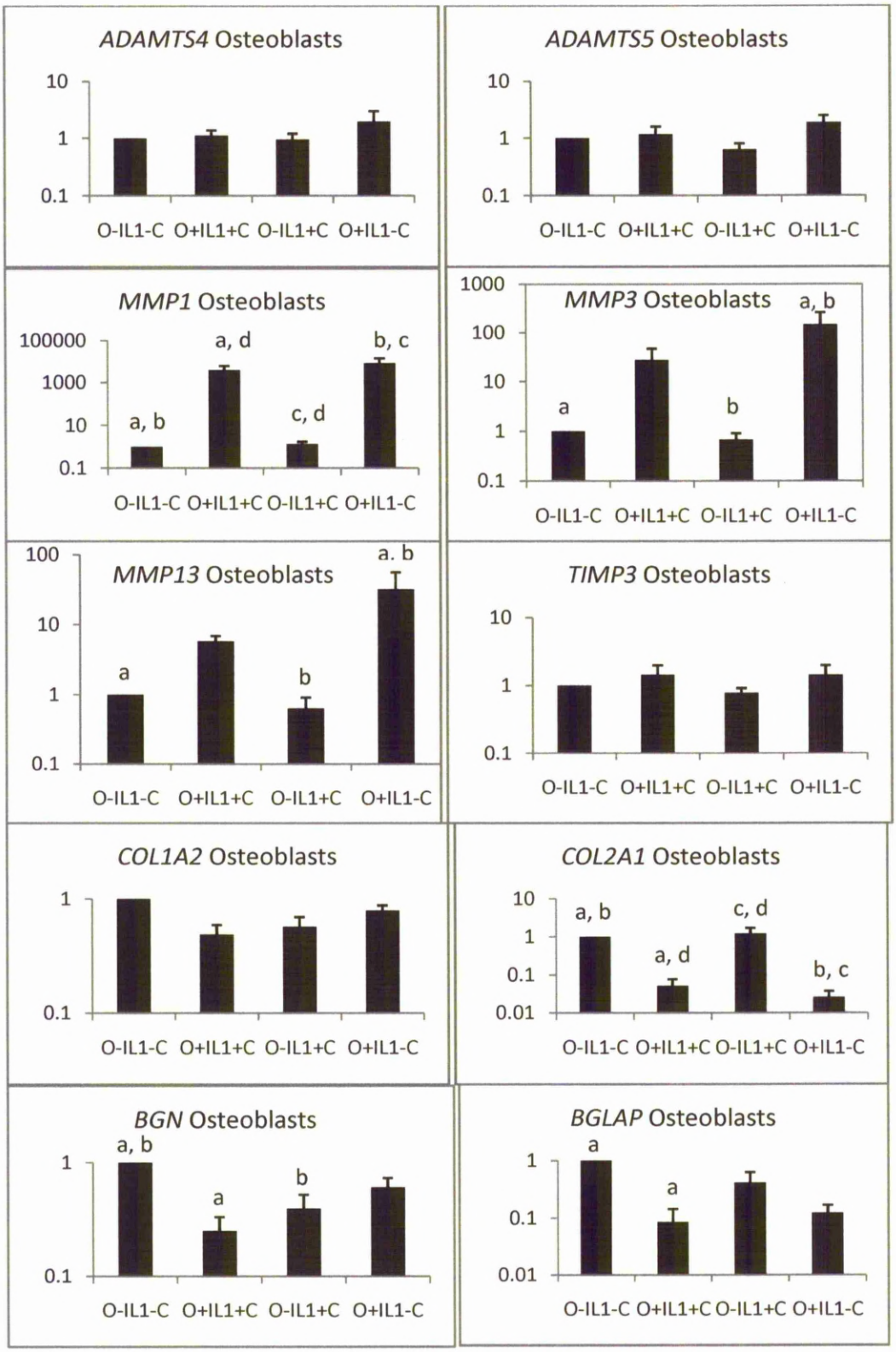


Figure 3.3a-j: Gene expression of osteoblasts shown as the log of fold differences compared to osteoblasts grown in monolayer (O-IL-C). Error bars indicate standard error. O-IL1-C = osteoblasts; O+IL1+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; O+IL1-C = osteoblasts pre-

treated with interleukin 1β. Groups with significant differences in gene expression are shown with letters on the graphs and corresponding P values are shown in Table 3.2.

3.3.3.2.1 Effect on osteoblasts of IL1β pre-treatment and co-culture with cartilage explants in comparison to negative control (O-IL1-C)

Comparing O+IL1+C osteoblasts to O-IL1-C osteoblasts there was; increased expression of *MMP1* (P=0.0001); decreased expression of *COL2A1* (P=0.0004), *BGN* (P=0.002) and *BGLAP* (P=0.005); and no significant difference in expression of *ADAMTS4, ADAMTS5, TIMP3, MMP3, MMP13* or *COL1A2*.

3.3.3.2.2 Effect of IL1β pre-treatment on osteoblasts co-cultured with cartilage explants

Comparing O+IL1+C to O-IL1+C osteoblasts there was; increased expression of *MMP1* (P=0.0001); decreased expression of *COL2A1* (P=0.0004); and no significant difference in expression of *MMP3, MMP13, ADAMTS4, ADAMTS5, TIMP3, COL1A2, BGN* and *BGLAP*.

3.3.3.2.3 Effect of co-culture with cartilage explants on osteoblasts pre-treated with IL1β

Comparing O+IL1+C to O+IL1-C osteoblasts there was no significant difference in expression of any of the genes examined (*ADAMTS4, ADAMTS5, MMP1, MMP3, MMP13, TIMP3, COL1A2, COL2A1, BGN* or *BGLAP*).

3.3.3.2.4 Effect of co-culture with cartilage explants on osteoblasts not pre-treated with IL1β

Comparing O-IL1+C to O-IL1-C osteoblasts there was; decreased expression of *BGN* (P=0.005) and no significant difference in expression of *ADAMTS4, ADAMTS5, MMP1, MMP3, MMP13, TIMP3, COL2A1, COL1A2* or *BGLAP*.

3.3.4 Fluorometric Activity Assay for MMP13

MMP13 activity of the culture media was measured in fluorescence units. Total MMP13 (active and inactive forms) is as shown in Figure 3.4 and Active MMP13 is as shown in Figure 3.5. Average

background count (calculated from fluorescence of culture media) was subtracted from the readings. P values showing the significance of differences in MMP13 activity between culture conditions are as shown in Table 3.3. The total MMP13 (active and inactive forms) was significantly greater in the media harvested from cartilage explants grown with osteoblasts pre-treated with IL1β as compared to cartilage explants grown with osteoblasts not pre-treated with IL1β. However, there was no significant difference in the levels of active MMP13 in the media harvested from the co-cultures with osteoblasts pre-treated or not with IL1β, with both co-cultures having elevated levels of MMP-13 as compared to the negative control (cartilage explant) and positive control (cartilage explant treated with IL1β).

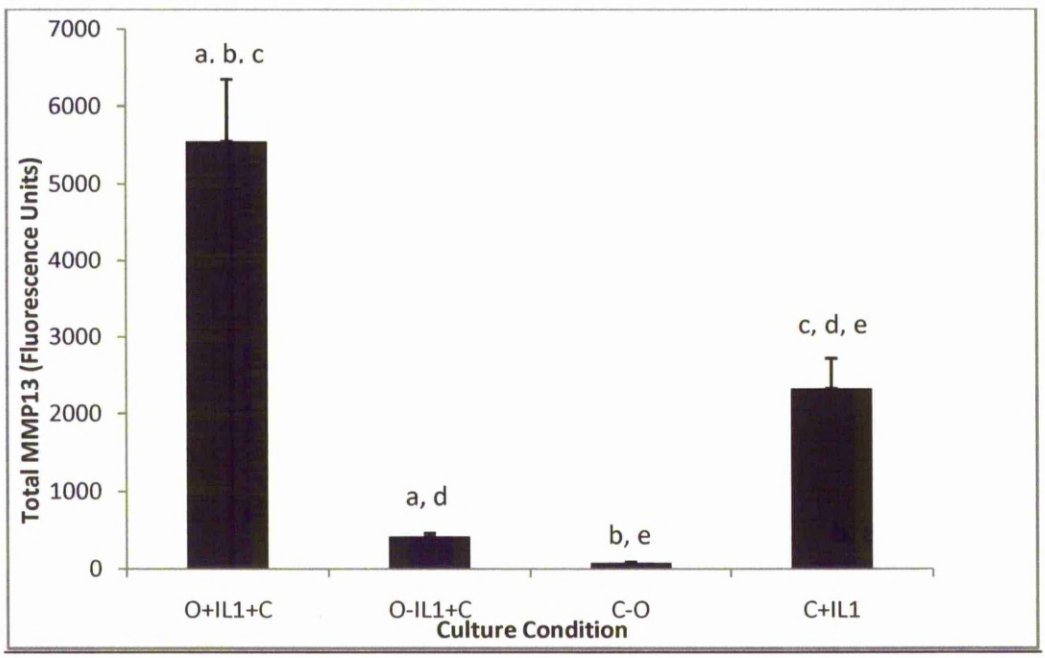


Figure 3.4: Total MMP13 (inactive and active forms of MMP13). Error bars indicate standard error. Groups with significant differences in MMP13 activity are shown with letters on the graphs and corresponding P values are shown in Table 3.3.
O+IL1+C = Osteoblasts pre-treated with interleukin 1β and co-cultured with cartilage explants
O-IL1+C = Osteoblasts not pre-treated with interleukin 1β and co-cultured with cartilage explants
C-O = Cartilage explants alone, without osteoblasts
C+IL1 = Cartilage explants treated with interleukin 1β

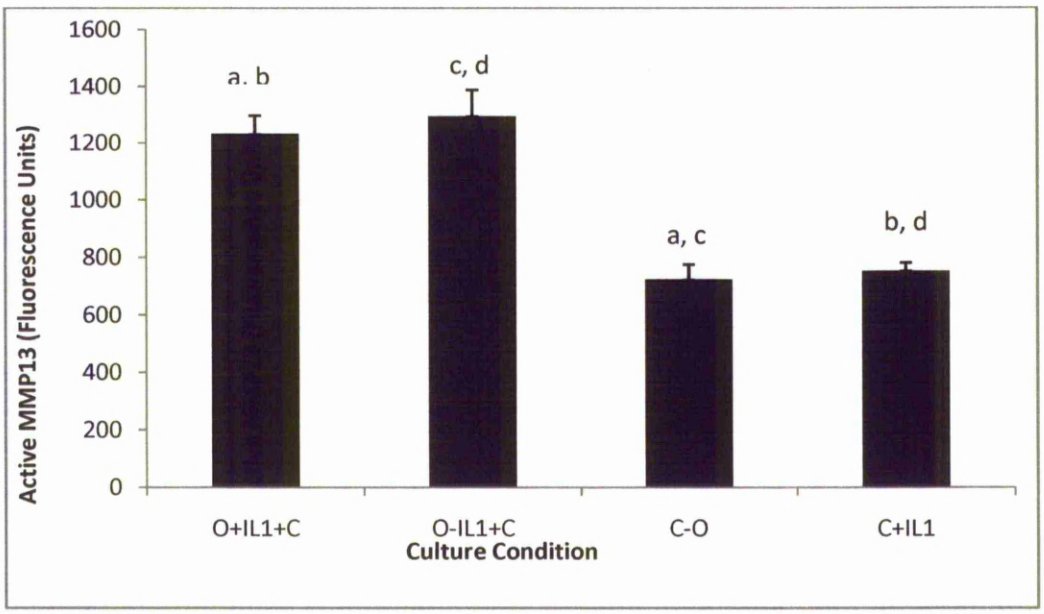


Figure 3.5: Total active MMP13 (without addition of APMA). Error bars indicate standard errors. Groups with significant differences in MMP13 activity are shown with letters on the graphs and corresponding P values are shown in Table 3.3.
O+IL1+C = Osteoblasts pre-treated with interleukin 1β and co-cultured with cartilage explants
O-IL1+C = Osteoblasts not pre-treated with interleukin 1β and co-cultured with cartilage explants
C-O = Cartilage explants alone, without osteoblasts
C+IL1 = Cartilage explants treated with interleukin 1β

MMP13 Activity	**Culture Condition, Significance of Differences in MMP13 Activity Between Groups and Increase (↑) or Decrease (↓) in MMP Activity (row compared to column)**			
Total MMP13 Activity (+APMA)		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P<0.0001↑	P = 0.0003↑	P<0.0001↑
	O-IL1β+C	P = 0.2	P = 0.0002↓	
	C+IL1β	P<0.0001↑		
Active MMP13 (-APMA)		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P<0.0001↑	P = 0.0001↑	P = 0.5
	O-IL1β+C	P<0.0001↑	P<0.0001↑	
	C+IL1β	P = 0.8		

Table 3.3: Statistical significance of differences in MMP13 activity between groups in media collected from different culture conditions. Calculated using mixed effects linear regression, significance level P≤0.05.
C-O= cartilage; O+IL1β+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1β+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; C+IL1β = cartilage treated with interleukin 1β

3.3.5 Glycosaminoglycan Spectrophotometric Analysis

Cumulative GAG release data was expressed as both a percentage of the total available GAG released into the media (as shown in Figure 3.6) and as GAG loss into the media per μg DNA of the papain digest (as shown in Figure 3.7). Significance of differences in GAG release between culture conditions at each time point as calculated using mixed effects linear regression is shown in Tables 3.4 and 3.5. When expressed as GAG loss into the media as a percentage of total GAG in the system, there was a significant increase in GAG release between O+IL1+C and the cartilage negative control at times 48 hours, 96 hours and 144 hours. By time 144 hours, the GAG release from O-IL1+C was also significantly increased as compared to the negative control. When expressed as GAG loss into the media per μg DNA, there was no significant difference in GAG release between either O+IL1+C or O-IL1+C and C-O at any time point. The positive control (C+IL1) had a significant increase in GAG release compared to all other culture conditions at all time points.

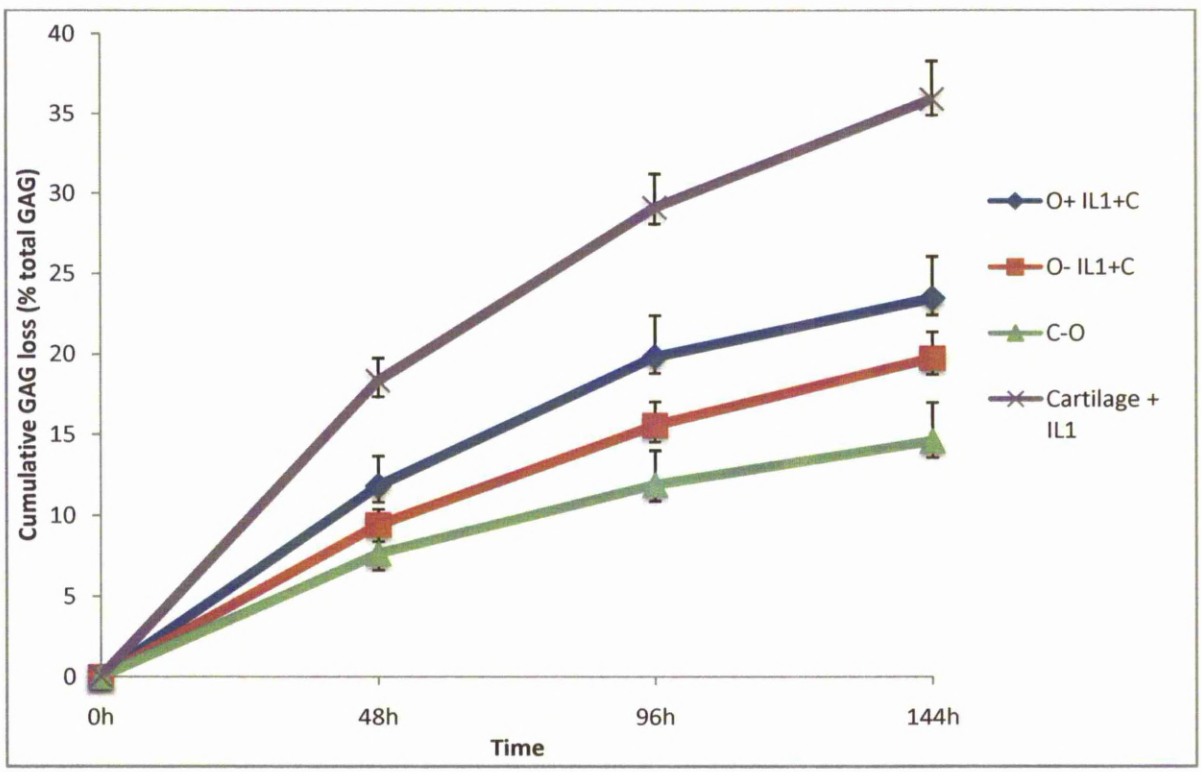


Figure 3.6: Cumulative GAG release over 144 hours in each culture condition expressed as percentage release of total GAG into the media.

GAG release	Culture Condition and Significance of Differences in GAG Release Between Groups and Increase(↑)or Decrease (↓) in GAG Release (row compared to column)			
48 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P = 0.03↑	P = 0.001↓	P = 0.2
	O-IL1β+C	P = 0.4	P<0.0001↓	
	C+IL1β	P<0.0001↑		
96 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P = 0.001↑	P = 0.002↓	P = 0.2
	O-IL1β+C	P = 0.05	P < 0.0001↓	
	C+IL1β	P<0.0001↑		
144 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P = 0.0008↑	P = 0.0004↓	P = 0.3
	O-IL1β+C	P = 0.02↑	P < 0.0001↓	
	C+IL1β	P<0.0001↑		

Table 3.4: Statistical significance of differences between groups in GAG release (expressed as percentage of total GAG) in media collected from different culture conditions. Calculated using mixed effects linear regression and significance level set at P≤0.05.
C-O= cartilage; O+IL1β+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1β+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; C+IL1β = cartilage treated with interleukin 1β

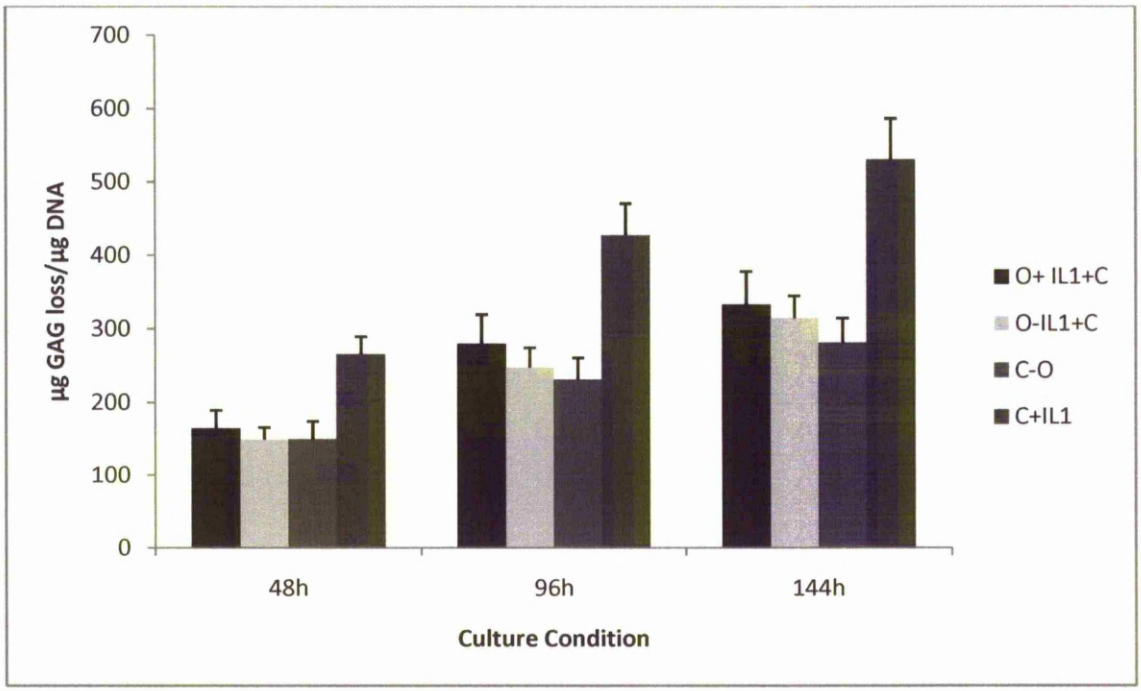


Figure 3.7: Cumulative GAG release over 144 hours in each culture condition expressed as µg GAG in the media per µg of DNA in the papain digest. Error bars indicate standard errors.
O+IL1+C = Osteoblasts pre-treated with interleukin 1β and co-cultured with cartilage explants
O-IL1+C = Osteoblasts not pre-treated with interleukin 1β and co-cultured with cartilage explants
C-O = Cartilage explants alone, without osteoblasts
C+IL1 = Cartilage explants treated with interleukin 1β

GAG release	Culture Condition and Significance of Differences in GAG Release Between Groups and Increase(↑)or Decrease (↓) in GAG Release (row compared to column)			
48 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P=0.6	P=0.003↓	P=0.6
	O-IL1β+C	P=1	P=0.0008↓	
	C+IL1β	P=0.0008↑		
96 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P=0.3	P=0.005↓	P=0.5
	O-IL1β+C	P=0.7	P=0.0009↓	
	C+IL1β	P=0.0003↑		
144 hours		C-O	C+IL1β	O-IL1β+C
	O+IL1β+C	P=0.4	P=0.002↓	P=0.8
	O-IL1β+C	P=0.6	P=0.0008↓↑	
	C+IL1β	P=0.0002↑		

Table 3.5: Statistical significance of differences between groups in GAG release (expressed as µg GAG/µg DNA in papain digest) in media collected from different culture conditions Calculated using mixed effects linear regression and significance level set at P≤0.05..
C-O= cartilage; O+IL1β+C = co-culture of osteoblasts pre-treated with interleukin 1β and cartilage; O-IL1β+C = co-culture of osteoblasts not pre-treated with interleukin 1β and cartilage; C+IL1β = cartilage treated with interleukin 1β

3.4 Discussion

With the series of experiments described, it has been shown that osteoblasts derived from normal equine subchondral bone when pre-treated with IL1β can induce an alteration in the phenotype of the chondrocytes of equine cartilage grown in co-culture without any direct communication between the cells. This would suggest the presence of soluble factors in the media, produced by the osteoblasts which are capable of inducing an effect in the gene expression of the chondrocyte.

Similar effects have been shown before, initially by Westacott et al. (1997) who demonstrated that media from cultures of primary osteoblasts obtained from OA joints increased GAG release from normal cartilage. Subsequently Hilal et al. (1999; 1998) showed that *in vitro* culture of osteoblasts prepared from human OA subchondral bone plates showed an altered phenotype with an alteration of the uPA/plasmin system activity and IGF-1 levels. Sanchez et al. (2005a, b) then showed that subchondral osteoblasts were capable of inducing phenotypic changes in human osteoarthritic chondrocytes grown in alginate beads in a co-culture system, and furthermore that alterations in aggrecan and metalloproteinase expression in chondrocytes could be mimicked by pre-treating osteoblasts obtained from non-sclerotic areas of OA bone with interleukin-6, -1β and oncostatin M. This is, however, the first time that these effects have been shown in osteoblasts obtained from normal bone in any species and in any form of osteoblast/chondrocyte co-culture in the equine.

An *in vitro* equine co-culture model consisting of non-osteoarthritic osteoblasts and cartilage explants has been developed in an attempt to minimise the variation that may be introduced to the system by pre-existing disease. The model was simplified by stimulating the osteoblasts with IL1β only and results of initial experiments to validate response of equine osteoblasts to IL1β are shown in a Chapter 2 (Culture of Equine Osteoblasts and Response to Interleukin 1β).

It has been shown previously that chondrocytes lose their phenotype in monolayer culture (von der Mark et al. 1977) and subsequently various culture systems have been developed which attempt to maintain chondrocyte phenotype including three-dimensional culture in agarose (Benya and Shaffer 1982), alginate (Stevens et al. 2004) and collagen (Takahashi et al. 2007) and explant cultures (Plaas and Sandy 1993). The effects of various culture models on phenotypic stability have also been investigated (Stewart et al. 2000) with the conclusion that aggregate and pellet culture models supported matrix protein gene expression profiles more reflective of *in vivo* levels, with expression of matrix protein genes being consistently depressed in explant cultures. In the designing of this co-culture system, it was decided to use cartilage explants as opposed to chondrocytes in alginate beads. This experimental design allowed the investigation not only of alterations in chondrocyte phenotype at mRNA level, but also allowed investigation of GAG release from the cartilage explant matrix as effected by treated osteoblasts. Usage of cartilage explants was possible in this equine model of osteoarthritis as there is a much greater availability of large quantities of normal tissue as compared to the human where available tissue tends to be sparse and diseased as a result of harvesting from joint prosthesis surgery.

Alterations in gene expression after direct application of IL1β to equine chondrocytes in suspension (Richardson and Dodge 2000) and monolayer (David *et al.* 2007) cultures have been reported previously, however there is no published data on the effects of IL1β on equine chondrocytes in explant culture. Richardson and Dodge (2000) showed a decrease in expression of type-II collagen, coupled with a marked increase in expression of *MMP1* and *MMP3* in equine chondrocytes in response to IL1β. Interestingly, in my experiments described here, similar alterations in mRNA expression to those described by Richardson and Dodge (2000) were seen in equine chondrocytes in explant culture effected via osteoblasts pre-treated with IL1β and also via direct application of IL1β. In comparison to the results of Richardson and Dodge (2000) who showed a slight decrease in *ACAN* mRNA and minimal effects on *BGN* expression in chondrocytes co-cultured with IL1β, I showed a

significant reduction in the expression of *BGN* and no significant effect on expression of *ACAN* after direct application of IL1β to cartilage explants and in chondrocytes co-cultured with IL1β pre-treated osteoblasts. These differences may be explained by differences in techniques utilised as Richardson and Dodge (2000)used suspension chondrocyte cultures and Northern blotting to detect mRNA while I utilised explant cultures and quantitative RT-PCR.

Our observed reduction in *BGN* expression in chondrocytes in the co-culture system and after direct application of IL1β may indicate that osteoblasts are able to drive an osteoarthritic phenotype in chondrocytes, whereby synthesis of non-collagenous matrix proteins is reduced. It may also have been expected to see a reduction in *ACAN* expression in chondrocytes after both direct application of IL1β and on co-culture with IL1β pre-treated osteoblasts, and the lack of effect on *ACAN* expression differs from the findings of Sanchez et al. (Sanchez *et al.* 2005a). This may possibly be explained by both species difference as I used equine tissue as opposed to human, and also because I used normal as opposed to osteoarthritic tissue. The finding that there was no significant difference in expression of *ACAN* in chondrocytes grown with untreated osteoblasts is consistent with those of Jiang et al. (2005) who showed the same effect using an osteoblast and chondrocyte micromass model of co-culture.

Chondrocyte and chondrogenic phenotype may be considered to be related to differential expression of cartilage specific *COL2A1* and previously it has been shown that IL1β decreases *COL2A1* production when IL1β is directly applied to human chondrocytes in culture (Goldring et al. 1988). Similarly, a reduction in *COL2A1* expression was observed in equine chondrocytes both after direct application of IL1β to explants and after co-culture with IL1β pre-treated osteoblasts. I found no alteration in chondrocyte *COL1A2* expression, which may not be surprising given that Goldring & Krane (1987) had previously shown that there was an increase in collagen type –I expression only when ambient levels of PGE_2 are suppressed by indomethacin.

The significant increases in expression of the proteolytic enzymes *MMP1, MMP3* and *ADAMTS4* and a trend towards an increase in *MMP13* expression in chondrocytes grown with IL1β pre-treated osteoblasts suggests that the osteoblasts are able to drive an osteoarthritic phenotype in the chondrocytes, and provides further evidence of the role of subchondral bone in OA. The fact that expression of these key proteolytic genes was not increased in chondrocytes co-cultured with osteoblasts not pre-treated with IL1β confirms that this is not an effect of co-culture alone.

Although a significant increase in *ADAMTS5* expression was observed in chondrocytes from cartilage explants treated directly with IL1β, the actual fold difference was small. Under the co-culture conditions, there was a lack of alteration in *ADAMTS5* expression in chondrocytes. This may be surprising given recent findings regarding the importance of ADAMTS5 as the major aggrecanase in mouse cartilage (Stanton et al. 2005). However, there appear to be species differences with regard to the importance of ADAMTS5 in the pathogenesis of OA. In the human, both ADAMTS4 and -5 have been found to be implicated in aggrecan degradation (Song et al. 2007) and there is evidence for complex post-transcriptional control of ADAMTS5 (Bondeson *et al.* 2008). The situation in equine cartilage is less clear, with a lack of published data.

The effect on gene expression of pre-treatment of osteoblasts grown in monolayer with IL1β compared to osteoblasts grown in monolayer, but not pre-treated with IL1β has been shown and discussed more fully in a previous chapter, but briefly there was increased expression of *MMP1, MMP3* and *MMP13*; decreased expression of *COL2A1*; and no difference in expression of *ADAMTS4, ADAMTS5, TIMP3, COL1A2, BGN or BGLAP*. When expression of the same panel of genes were compared in osteoblasts pre-treated or not with IL1β and grown with cartilage explants, a similar pattern of gene expression was seen, although the increases in *MMP3* and *MMP13* expression did not quite reach significance after correction for multiple comparisons . This would suggest that co-

culture with cartilage explants does not greatly alter the effect of pre-treatment with IL1β on osteoblast phenotype.

The effect of co-culture with cartilage explants on osteoblast phenotype was further assessed by comparing gene expression between osteoblasts pre-treated with IL1β cultured with and without cartilage explants and also non IL1β pre-treated osteoblasts cultured with and without cartilage explants (i.e. O+IL1+C compared to O+IL1-C and O-IL1+C compared to O-IL1-C). There were limited effects on gene expression when the groups were compared in this manner in both the IL1β pre-treated osteoblasts and in the non pre-treated osteoblasts, indicating that the effect of co-culture with cartilage explants on osteoblast phenotype without any other manipulation is small.

The validation of differentiation of the equine osteoblast in cell culture and response to IL1β is addressed more fully in Chapter 2 (Culture of Equine Osteoblasts and Response to Interleukin 1β), however it is worth noting that pre-treatment of the osteoblasts with IL1β in this co-culture system resulted in a significant decrease in *BGLAP* expression when O+IL1β+C osteoblasts were compared to O-IL1β-C osteoblasts. Osteocalcin in frequently used as a marker of osteogenic phenotype and of the mature osteoblast (Lynch et al. 1995) and this finding may suggest that the osteoblasts were dedifferentiating after treatment. However, I was satisfied with differentiation of the osteoblasts in the co-culture given that osteocalcin was being expressed, the reduced *BGLAP* expression was observed only in one comparison of culture conditions and had a borderline significance level, *COL1A2* was expressed in osteoblasts under all culture conditions and osteoblast phenotype was also confirmed by production of alkaline phosphatase and positive staining with Alizarin Red and von Kossa stains.

Depletion of proteoglycans from the extracellular matrix of articular cartilage is a common initial change in joint disease, followed by subsequent degradation of the collagen fibrils, with proteinases

being key to this degradation process. Increases in gene expression of *MMP1, MMP3, MMP13* and *ADAMTS4* by chondrocytes had been identified. Traditionally MMP1 and MMP13 are classed as collagenases and MMP3 as a stromelysin (Cawston and Wilson 2006). Collagenases have the ability to cleave fibrillar collagens at a specific site, producing characteristic three-quarter and one-quarter sized fragments. MMPs have also been shown to have the ability to cleave aggrecan (Sandy 2006) at sites distinct from those utilised by the recently discovered ADAMTs group of enzymes In comparison (Nagase and Kashiwagi 2003). MMP3 has the ability to degrade proteoglycans and also activates latent collagenases (Cawston and Wilson 2006).

Depletion of matrix proteoglycan was observed in this co-culture model, shown by increased levels of GAG release from the cartilage explant matrix into the cell culture media on co-culture with IL1β pre-treated osteoblasts compared to the cartilage explant alone at T=48, T=96 and T=144 hours. However, at T=144 hours there was also an increase in GAG release in the cartilage explants grown with untreated explants. The effect of co-culture with IL1β pre-treated osteoblasts on the degradation of cartilage was therefore not as dramatic as may have been expected from the alterations in gene expression of the proteinases. This may be explained by the fact that the relationship between mRNA expression of MMPs, secretion of the latent pro-peptide and activation of the proMMP into its active and physiologically important form is complex and by no means linear. In addition, there is the added possibility of inhibition of MMP activity by TIMPs.

In investigation of cartilage matrix breakdown, I looked also at MMP13 protein levels (active and total) in cell culture media. It was shown that although there was a marked increase in total MMP-13 in the cell media from pre-treated osteoblasts co-cultured with cartilage explants, this MMP-13 was not necessarily in its active form and in fact, levels of active MMP13 protein were similar within both co-culture groups (O+IL1+C and O-IL1+C), with both groups having increased active MMP-13 levels compared to the C-O negative control. It may have been interesting to look further at

collagenolysis using techniques such as hydroxyproline assays or identification of cleavage by measurement of COL2 neoepitopes. Also, the apparent lack of correlation between increased expression of proteinases and the less dramatic evidence of GAG release from the cartilage matrix may have been further investigated using antibodies to identify various neoepitopes on aggrecan degradation products.

3.5 Conclusion

A repeatable co-culture model for equine osteoblasts and chondrocytes simulating osteochondral disease has been developed. I demonstrated alterations in the gene expression of osteoblasts pre-treated with IL1β, and that these cells were capable of inducing associated alterations in the gene expression of chondrocytes in co-culture. There was some evidence of cartilage matrix breakdown characterised by GAG release from the cartilage explants in these co-cultures. Matrix breakdown may have been attenuated however as the majority of the MMP-13 being produced as a result of increased gene expression was not cleaved to its active form.

3.6 References

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Chapter 3 Appendix A

Does ITS+ Prevents Cartilage Breakdown?

Introduction

Choice of cell culture media is always a contentious subject, but even more so in extended co-culture experiments whereby two cell types must be supported for a lengthy period while at the same time the investigator tries to avoid the use of any media supplementation which may interfere with the experiment.

Defined media for cell culture of varying complexity are commercially available, but all are composed of essential amino acids, vitamins, salts and glucose (Freshney 1994). Serum from a variety of sources e.g. calf, foetal bovine, horse and human is widely added to basic cell culture media to support cell growth by providing a source of protein, polypeptides, hormones, nutrients and minerals. However, the use of serum may have disadvantages in that as a biological substance, the exact components of the media are unknown and may vary from batch to batch thus introducing variability into the experimental design. Furthermore, serum may contain growth factors such as TGF-β which will have a negative effect on cell proliferation. To circumvent these issues, the use of serum free media has been proposed and with some cell types this is successful. However, growth is often slower in serum-free media and cells may require support using more complex media which also contain organic supplements such as proteins, peptides, nucleosides, citric acid cycle intermediates, pyruvate and lipids. It is possible also to use a basic cell media and supplement this with a commercially available and defined serum substitute (Freshney 1994). ITS+ (Sigma-Aldrich, Dorset, UK) is one such commercially available serum substitute containing 1.0mg/ml insulin, 0.55 mg/ml human transferrin and 0.5μg/ml sodium selenite.

As described in the preceding chapter, a protocol was adapted from that of Sanchez et al. (2005) such that a cell culture media containing DMEM and 1%ITS+ was used for the co-culture step. Results using this experimental design showed alterations in gene expression of both chondrocytes and osteoblasts and alterations in MMP-13 assays, with evidence of support of osteoblast phenotype based on cell staining and alkaline phosphatase assays. However, the levels of GAG release from the cartilage explants were extremely low, both in the co-cultures and in the positive control whereby interleukin 1β (IL1β) was applied directly to cartilage explants. A possible reason for this was thought to be the addition of ITS+ to the culture medium, and the following experiment was performed to determine 1) if ITS+ was preventing cartilage breakdown and 2) to assess the effects of various concentrations of ITS+ and FBS (foetal bovine serum) in the culture medium on cartilage breakdown driven by IL1β.

Materials and Methods

Cartilage explants were obtained from the metacarpophalangeal joints of horses euthanased at The University of Liverpool Equine Hospital for reasons unrelated to orthopaedic disease/osteoarthritis and where owners had given informed consent for use of samples from their horse, or from healthy horses euthanased at a local abattoir, as described previously. Each joint was grossly free from osteoarthritis. Explants were cut into small pieces (5mm x 5mm approx) and left overnight at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml amphotericin B. Cartilage explants were then washed and utilised in the experiment. All reagents were supplied by Sigma-Aldrich (Dorset, UK).

In the first part of the experiment, the effect of different levels of ITS+ in the media of cartilage explants on treatment with interleukin 1β (IL1β) was assessed. Experiments were performed in triplicate using 5 separate donors with explants being cultured in DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml amphotericin B with the addition of:

1) 1% ITS+
2) 1% ITS+ and 10ng/ml IL1β
3) 0.1% ITS+ and 10ng/ml IL1β
4) 0.01% ITS+ and 10ng/ml IL1β
5) 0% ITS+ (serum free media negative control)
6) 0% ITS+ and 10ng/ml IL1β

In the second part of the experiment, the effect of different levels of serum in the media was compared to the effect of ITS+ on treatment with IL1β. Experiments were performed in triplicate using 3 separate donors and in the same basic media as above with the addition of:

1) 1%ITS+ and 10ng/ml IL1β
2) 1% ITS+
3) 10ng/ml IL1β
4) Nothing (serum free media negative control)
5) 1% foetal bovine serum (FBS) and 10ng/ml IL1β
6) 1% FBS
7) 10% FBS and 10ng/ml IL1β
8) 10% FBS

Cultures were harvested at 5 days and glycosaminoglycan spectrophotometric analysis was performed on the media and cartilage explants to quantify levels of glycoasminoglycan (GAG). Harvested cartilage explants were digested by overnight papain incubation at 60°C. Aliquots of media (40μL) and of digested explants (2μL diluted with 38μL H_2O) were analysed for glycosaminoglycan (GAG) content using the 1.9-dimethylene blue (DMMB) labelling technique as before. Sulphated GAG concentrations were determined by absorbance readings on a Multiskan EX photometric multiplate absorbance reader (Thermo Electron Corp, Vantaa, Finland) at 570nm

relative to a shark chondroitin sulphate C standard curve (0 to 70μg/ml). Cumulative GAG release data was expressed as a percentage of the total available GAG in the system released into the media, such that:

$$\% \text{ GAG release} = \frac{\text{GAG in media}}{\text{GAG in media + GAG in explant}} \times 100$$

Descriptive statistical analysis was performed using Mintab (v.15; Minitab Inc., Pennsylvania) and differences between culture groups were detected using mixed effects linear regression (SPlus v. 6.1; TIBCO Software Inc., California), with significance being set at $P<0.05$.

Results

Percentages of GAG release in the first part of the experiment (the effect of different ITS+ culture media concentrations on IL1β driven GAG release from cartilage explants) are shown for each donor in Figure 1 and as an average of all donors in Figure 2. Values of mean GAG loss and standard errors are given in Table 1, and statistical significance of differences between groups are shown in Table 2.

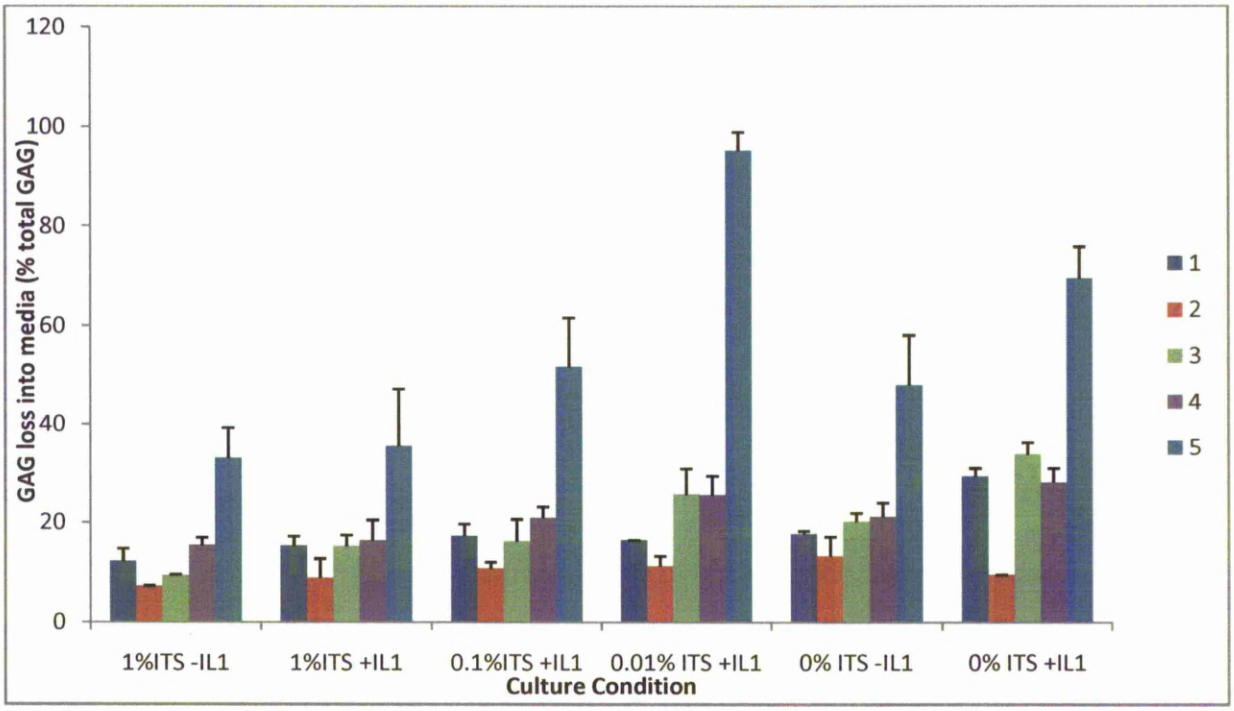

Figure 1: GAG loss into media expressed as a percentage of total GAG in the culture system for each of the donors in each of the culture conditions in part 1 of the experiment. Error bars indicate standard errors.

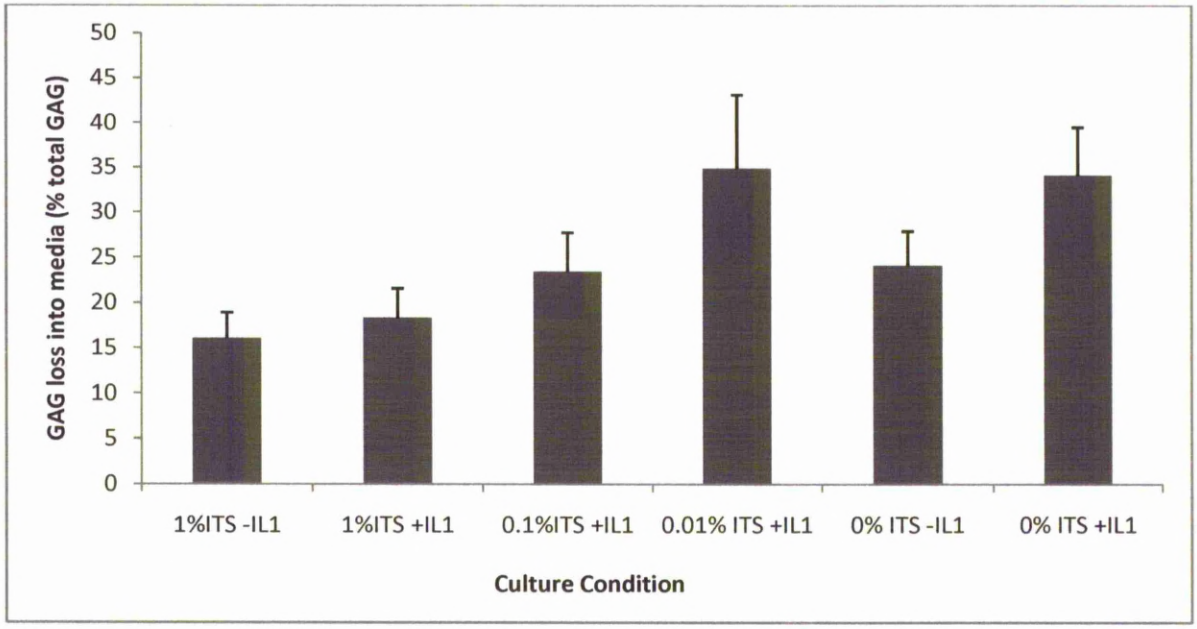

Figure 2: GAG loss into media expressed as a percentage of total GAG in the culture system for each of the culture conditions in part 1 of the experiment averaged for all donors. Error bars indicate standard errors.

Culture Condition: DMEM plus	Mean GAG loss; (Standard Error)
1% ITS+	16.1%; (+/- 2.9)
1% ITS+ and 10ng/ml IL1β	18.3%; (+/- 3.3)
0.1% ITS+ and 10ng/ml IL1β	23.4%; (+/- 4.3)
0.01% ITS+ and 10ng/ml IL1β	34.9%; (+/- 8.3)
0% ITS+	24.1%; (+/- 3.8)
0% ITS+ and 10ng/ml IL1β	34.1%; (+/- 5.4)

Table 1: GAG loss as a response to culture condition (+/- IL1β treatment in different ITS+ concentrations in media)

Culture Condition and Significance of Differences in GAG Release Between Groups					
	1% ITS+	0% ITS+ and IL1	0% ITS+	0.01% ITS+ and IL1	0.1% ITS+ and IL1
1% ITS+ and IL1	P=0.2	P<0.0001	P=0.006	P<0.0001	P=0.04
0.1% ITS+ and IL1	P=0.0007	P=0.0015	P=0.5	P=0.02	
0.01% ITS+ and IL1	P<0.0001	P=0.3	P=0.1		
0% ITS+	P=0.0001	P=0.01			
0% ITS+ and IL1	P<0.0001				

Table 2: Statistical Significance between Culture Groups for GAG Release with IL1β Treatment at Different ITS+ Concentrations

In the absence of interleukin treatment, addition of 1% ITS+ to the culture medium was shown to result in significantly less GAG release than when explants were cultured in basic serum free DMEM without supplementation with ITS+ (0% ITS+). ITS+ was shown to protect the explants against GAG release in response to IL1β treatment in a dose-dependent manner.

Results of the second part of the experiment (the effect of different concentrations of FBS in the culture media as compared to ITS+ on IL1β driven GAG release from cartilage explants) are shown for each donor in Figure 3 and as an average of all donors in Figure 4. Values of mean GAG loss and standard errors are given in Table 3, and statistical significance of differences between groups are shown in Table 4.

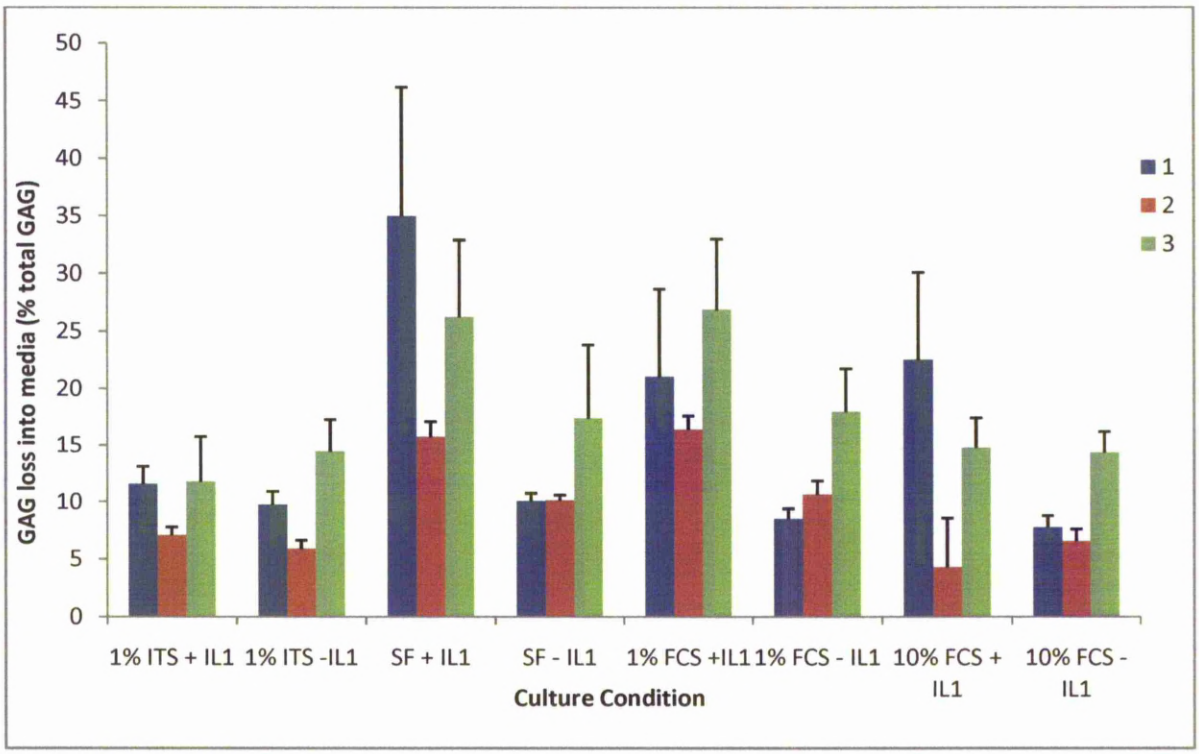

Figure 3: GAG loss into media expressed as a percentage of total GAG in the culture system for each of the donors in each of the culture conditions in part 2 of the experiment. Error bars indicate standard errors.

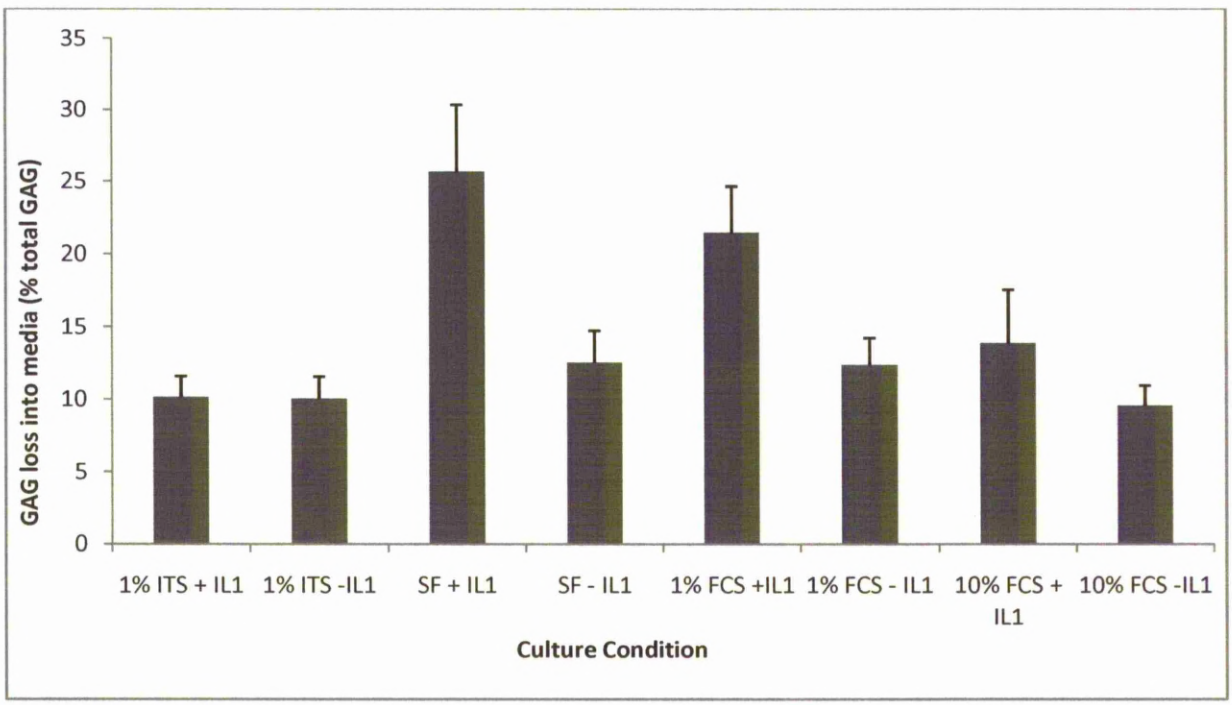

Figure 4: GAG loss into media expressed as a percentage of total GAG in the culture system for each of the culture conditions in part 2 of the experiment averaged for all donors. Error bars indicate standard errors.

Culture Condition: DMEM plus	Mean GAG loss; (Standard Error)
1% ITS+ and 10ng/ml IL1β	10.1% (+/- 1.5)
1% ITS+	10.0% (+/- 1.5)
10ng/ml IL1β	25.6% (+/- 4.7)
serum free media negative control	12.5% (+/- 2.2)
1% FBS and 10ng/ml IL1β	21.4% (+/- 3.2)
1% FBS	12.4% (+/- 1.9)
10% FBS and 10ng/ml IL1β	13.9% (+/- 3.7)
10% FBS	9.53% (+/- 1.4)

Table 3: GAG loss as a response to culture condition (+/- IL1β treatment in varying FCS concentrations and as a comparison to 1% ITS+)

Culture Condition and Significance of Differences in GAG Release Between Groups							
	1% ITS+ and IL1	10% FBS	10% FBS and IL1	1% FBS	1% FBS and IL1	SF (neg. control)	SF + IL1
1% ITS+	P=1	P=0.9	P=0.3	P=0.5	P=0.002	P=0.5	P<0.0001
IL1	P<0.0001	P<0.0001	P=0.002	P=0.0004	P=0.2	P=0.0005	
SF (neg. control)	P=0.5	P=0.4	P=0.7	P=1	P=0.01		
1% FBS and IL1	P=0.002	P=0.001	P=0.04	P=0.01			
1% FBS	P=0.5	P=0.4	P=0.7				
10% FBS and IL1	P=0.3	P=0.2					
10% FBS	P=0.9						

Table 4: Statistical Significance between Culture Groups for GAG Release with IL1ß Treatment at Varying FCS Concentrations and as a Comparison to 1% ITS+
FBS = foetal bovine serum; SF = basic serum free DMEM media; IL1 = interleukin 1ß

In the second set of experiments, the finding that addition of 1% ITS+ resulted in significantly less GAG release than culture in basic serum free DMEM was not repeated. However on treatment with IL1β, addition of 10% FBS to the culture medium was found to protect the explants against GAG release. Reducing the concentration of FBS to 1% resulted in a significant effect of IL1β treatment on GAG release.

Discussion

It was shown that addition of ITS+ to the basic serum-free DMEM culture medium at a concentration of 1% was capable of exerting an effect such that GAG release from the cartilage explants was significantly reduced in response to interleukin 1β treatment. With lower concentrations of ITS+ a similar effect was seen in a dose responsive manner. As stated previously, ITS+ is composed of insulin, transferrin and selenium. Insulin is a hormone which can affect cell metabolism via its effects on glucose metabolism and by activity via the insulin-like growth factor (IGF) receptor (Hewlett 1991). Transferrin is an iron-transport protein which can also act as a detoxifying agent by chelating heavy metals and selenium is a trace element which has been found to be required for cell growth (Hewlett 1991).

It is likely that the addition of ITS+ to the culture medium is most likely to decrease GAG release as a result of the increased levels of insulin. It is unclear whether ITS+ is exerting its effects via a decrease in GAG loss from the matrix or as a result of increased GAG production in the cartilage explant, as both would result in a decreased percentage of GAG loss from the system into the media. It has been postulated that insulin may exert an effect via the IGF receptor. IGF-1 appears to be one of the most important growth factors affecting the anabolism of cartilage (Martel-Pelletier *et al.* 1998) and has been shown to stimulate production of chondrocyte extracellular matrix components (Guenther *et al.* 1982; McQuillan *et al.* 1986). IL-1 is known to be a key cytokine in degradation of the cartilage matrix (Dingle *et al.* 1979; Saklatvala *et al.* 1984) and is also capable of suppressing synthesis of aggrecan and collagen by the chondrocyte (Dingle 1984). IGF-1 has also been shown to directly decrease both basal and cytokine (IL1 and TNF) stimulated degradation of proteoglycan in cartilage (Tyler 1989). Subsequently it was found that IGF-1 is able to reverse IL1β mediated degradation of aggrecan and repression of the aggrecan synthesis rate through up-regulation of the decoy receptor of IL1β, IL1 receptor II. In short therefore, it is thought that the addition of insulin in

ITS+ may have similar effects on cartilage metabolism and in reversing IL1 degradation as insulin-like growth factor I.

It is therefore considered inappropriate to add ITS+ to cell culture medium in co-culture experiments where cartilage degradation will be examined as a response. Subsequent experiments showed that addition of 10% FCS to the culture medium had a similar effect on prevention of cartilage breakdown as 1% ITS+. As the lower concentration of 1% FCS did not interfere with IL1 driven cartilage breakdown and had previously been shown to support osteoblast culture, this culture medium was therefore used for co-culture systems where glycosaminoglycan spectrophotometric analysis was to be performed on media and digests of cartilage explants.

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Chapter 3 Appendix B

GAG Release from Cartilage Explants in Response to Pre-Treated Osteoblasts is Mediated via the Chondrocyte

Introduction

It has been shown that osteoblasts pre-treated with interleukin 1β have increased expression of various proteolytic enzymes and are in turn capable of altering the expression of proteolytic enzymes and markers of matrix synthesis such as collagen–II and aggrecan in co-cultured chondrocytes. It has also been shown that there is an effect of co-culture of both pre-treated and untreated osteoblasts on cartilage breakdown of co-cultured live cartilage explants as measured by glycosaminoglycan release into the media.

It is interesting to consider to what degree the effect of osteoblasts on cartilage matrix proteolysis is effected via a direct response of soluble mediators produced by the osteoblasts transported in the media to breakdown the cartilage matrix, and to what degree cartilage breakdown is driven by soluble mediators produced by the osteoblasts acting on the chondrocyte, which in turn produce enzymes which result in cartilage breakdown.

Based on previous literature which has shown that matrix depletion occurs in the presence of supernatants from stimulated human blood monocytes and cartilage explants with live chondrocytes, but not cartilage explants with dead chondrocytes (Jasin and Dingle 1981), a means of doing this was to compare the response of cartilage explants containing live and dead chondrocytes to each of the co-culture conditions as described in the preceding chapter.

Materials and Methods

The experimental design of the co-culture was as described in the preceding chapter. Briefly osteoblasts were isolated from the subchondral bone of the metacarpophalangeal joint of horses grossly unaffected by osteoarthritis and differentiated for 10 days. At the end of osteoblast differentiation, cells were washed and either treated with 10ng/ml IL1β or left untreated for 24

hours. After pre-treatment, osteoblasts were again washed and then co-cultures set up with cartilage explants which were either live or had been killed by 3 freeze-thaw cycles in liquid nitrogen.

Culture groups were therefore:

1) Osteoblasts pre-treated with IL1β co-cultured with live cartilage explants (O+IL1+C L)
2) Osteoblasts pre-treated with IL1β co-cultured with dead cartilage explants (O+IL1+C D)
3) Osteoblasts not pre-treated with IL1β co-cultured with live cartilage explants (O-IL1+C L)
4) Osteoblasts not pre-treated with IL1β co-cultured with dead cartilage explants (O-IL1+C D)
5) Live cartilage explants cultured alone without osteoblasts (C L-O)
6) Dead cartilage explants cultured alone without osteoblasts (C D-O)
7) Live cartilage explants cultured with IL1β (C L+IL1)
8) Dead cartilage explants cultured with IL1β (C D+IL1)

The media used for this co-culture experiment was Dulbecco's modified Eagle medium (DMEM) supplemented with 1% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml amphotericin B as discussed in Appendix A. Co-cultures were continued for 144 hours with media being changed and collected at 48 hour intervals.

Cartilage explants were digested in papain as previously described and GAG content of the explant digest and of the media were measured using glycosaminoglycan spectrophotometric analysis and the 1.9-dimethylene blue (DMMB) labelling technique as before. Cumulative GAG release data was expressed as a percentage of the total available GAG in the system released into the media, such that:

$$\% \text{ GAG release} = \frac{\text{GAG in media}}{\text{GAG in media + GAG in explant}} \times 100$$

Descriptive statistical analysis was performed using Mintab (v.15; Minitab Inc., Pennsylvania) and differences between culture groups were detected using mixed effects linear regression (SPlus v. 6.1; TIBCO Software Inc., California), with significance being set at $P<0.05$.

Results

Average cumulative GAG release and average cumulative GAG release with standard errors over 144 hours are as shown in Figures 1 and 2. Mean GAG release and standard errors are shown in Table 1. The statistical significance of differences between GAG release in culture condition groups are as shown in Table 2.

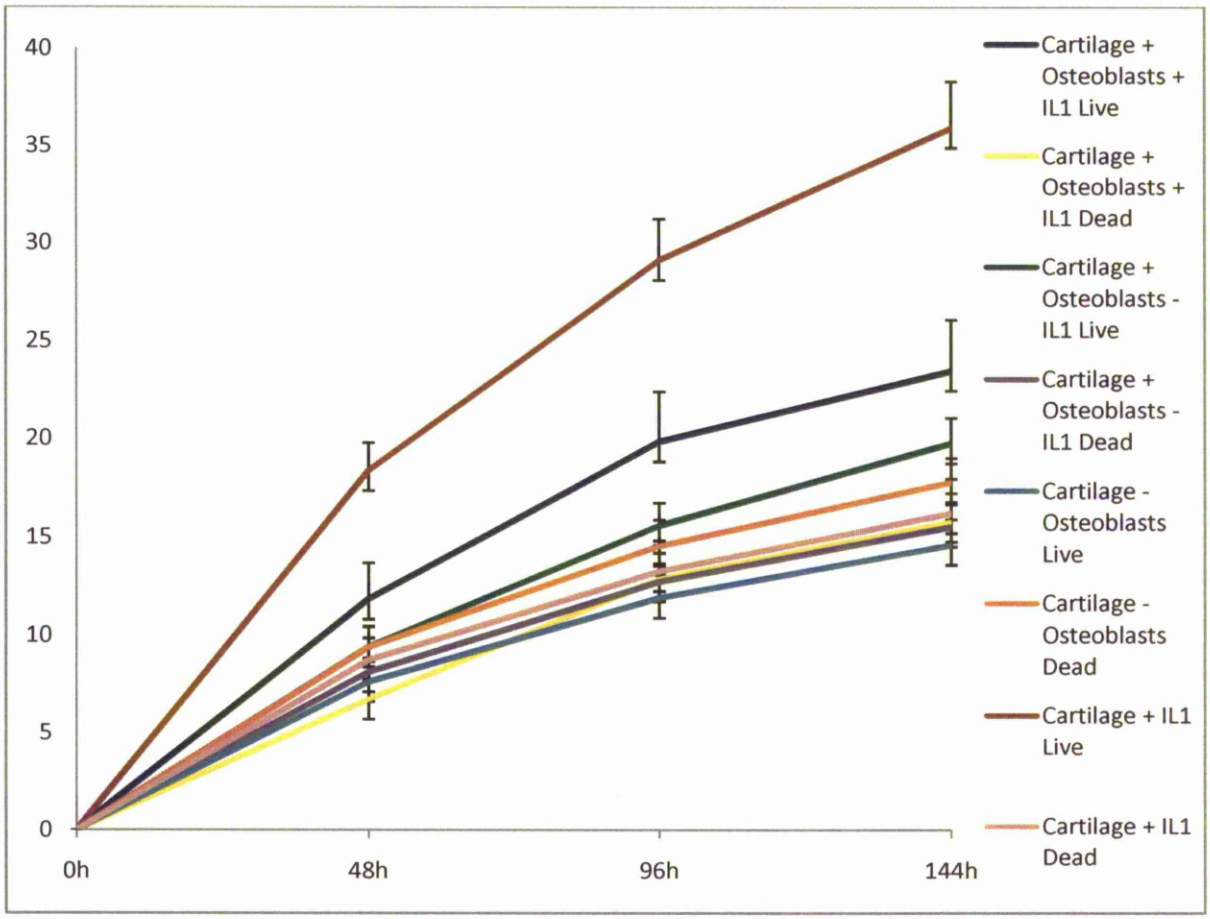

Figure 1: Average cumulative GAG release over 144 hours in each culture condition expressed as percentage release of total GAG into the media. Error bars show standard error.

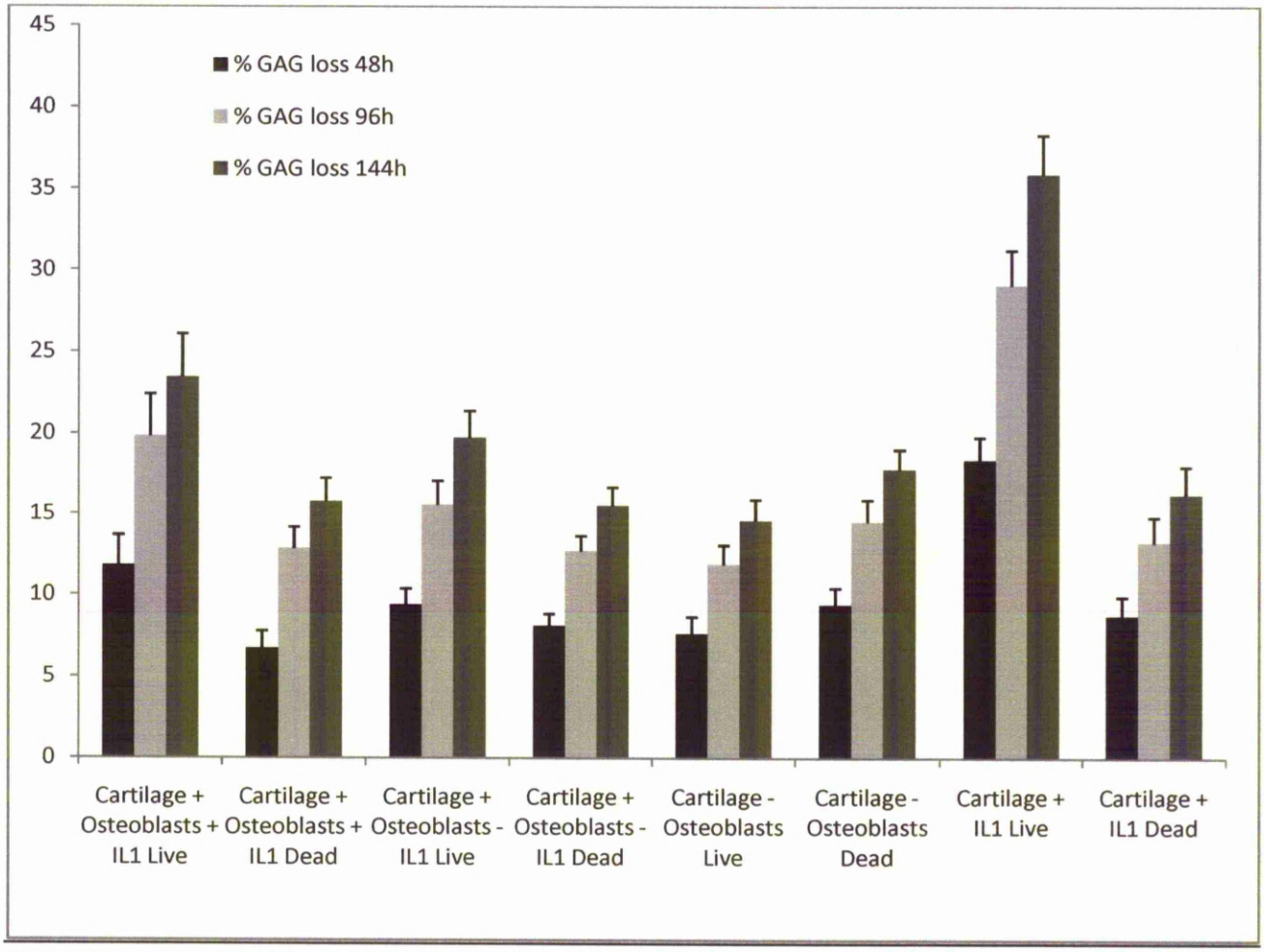

Figure 2: Average cumulative GAG release over 144 hours in each culture condition expressed as percentage release of total GAG into the media. Error bars indicate standard error.

Culture Condition	**Mean GAG loss; (Standard Error)**		
	T=48 hours	T=96 hours	T=144 hours
Live cartilage	7.6% (+/- 1.1)	11.9% (+/- 1.2)	14.6% (+/- 1.3)
Live cartilage treated with IL1β	18.4% (+/- 1.4)	29.1% (+/- 2.2)	35.9% (+/- 2.4)
IL1β pre-treated osteoblasts and live cartilage	11.8% (+/- 1.9)	19.8% (+/- 2.6)	23.5% (+/- 2.6)
Untreated osteoblasts and live cartilage	9.4% (+/- 1)	15.6% (+/- 1.5)	19.7% (+/- 1.7)
Dead cartilage	9.4% (+/- 1.1)	14.5% (+/- 1.4)	17.8% (+/- 1.3)
Dead cartilage treated with IL1β	8.73% (+/- 1.1)	13.2% (+/- 1.6)	16.2% (+/- 1.8)
IL1β pre-treated osteoblasts and dead cartilage	8.3% (+/- 1.1)	12.8% (+/- 1.3)	15.8% (+/- 1.5)
Untreated osteoblasts and dead cartilage	8.1% (+/- 0.7)	12.7% (+/- 1)	15.5% (+/- 1.2)

Table 1: GAG loss (expressed as percentage of total GAG lost into media) in each culture condition

Time	Culture Condition, Significance of Differences in GAG Release Between Groups and Increase (↑) or Decrease (↓) in GAG Release (row compared to column)							
48 hours		Cartilage + Osteoblasts + IL1 Live	Cartilage +IL1 Dead	Cartilage +IL1 Live	Cartilage Dead	Cartilage Live	Cartilage + Osteoblasts – IL1 Dead	Cartilage + Osteoblasts –IL1 Live
	Cartilage + Osteoblasts + IL1 Dead	P=0.1	P=0.8	P<0.0001↓	P=0.4	P=0.4	P=0.9	P=0.4
	Cartilage + Osteoblasts – IL1 Live	P=0.5	P=0.5	P=0.0001↓	P=0.9	P=0.1	P=0.4	
	Cartilage + Osteoblasts – IL1 Dead	P=0.1	P=0.9	P<0.0001↓	P=0.5	P=0.4		
	Cartilage Live	P=0.01↓	P=0.3	P<0.0001↓	P=0.1			
	Cartilage Dead	P=0.4	P=0.6	P=0.0001↓				
	Cartilage +IL1 Live	P=0.001↑	P<0.0001↑					
	Cartilage +IL1 Dead	P=0.2						
96 hours		Cartilage + Osteoblasts + IL1 Live	Cartilage +IL1 Dead	Cartilage +IL1 Live	Cartilage Dead	Cartilage Live	Cartilage + Osteoblasts – IL1 Dead	Cartilage + Osteoblasts –IL1 Live
	Cartilage + Osteoblasts + IL1 Dead	P=0.004↓	P=0.9	P<0.0001↓	P=0.3	P=0.6	P=0.9	P=0.1
	Cartilage + Osteoblasts – IL1 Live	P=0.2	P=0.2	P<0.0001↓	P=0.6	P=0.04↑	P=0.2	
	Cartilage + Osteoblasts – IL1 Dead	P=0.006↓	P=1	P<0.0001↓	P=0.4	P=0.5		
	Cartilage Live	P=0.0007↓	P=0.5	P<0.0001↓	P=0.1			
	Cartilage Dead	P=0.06	P=0.4	P<0.0001↓				
	Cartilage +IL1 Live	P=0.002↑	P<0.0001↑					
	Cartilage +IL1 Dead	P=0.006↓						
144 hours		Cartilage + Osteoblasts + IL1 Live	Cartilage +IL1 Dead	Cartilage +IL1 Live	Cartilage Dead	Cartilage Live	Cartilage + Osteoblasts – IL1 Dead	Cartilage + Osteoblasts –IL1 Live
	Cartilage + Osteoblasts + IL1 Dead	P=0.004↓	P=0.9	P<0.0001↓	P=0.3	P=0.5	P=1	P=0.06
	Cartilage + Osteoblasts – IL1 Live	P=0.3	P=0.08	P<0.0001↓	P=0.4	P=0.01↑	P=0.06	
	Cartilage + Osteoblasts – IL1 Dead	P=0.004↓	P=0.9	P<0.0001↓	P=0.3	P=0.6		
	Cartilage Live	P=0.0006↓	P=0.5	P<0.0001↓	P=0.09			
	Cartilage Dead	P=0.07	P=0.3	P<0.0001↓				
	Cartilage +IL1 Live	P=0.0003↑	P<0.0001↑					
	Cartilage +IL1 Dead	P=0.006↓						

Table 2: Statistical significance of differences between groups in GAG release (expressed as percentage of total GAG) in media collected from different culture conditions.
C = cartilage; O = osteoblasts; +IL1 = pre-treated with interleukin 1β; -IL1 = untreated

Discussion and Conclusion

The increased GAG release from cartilage explants co-cultured with osteoblasts pre-treated with IL1β has been reported and discussed in the main chapter. This finding is expanded here to show that when co-cultured with IL1β pre-treated osteoblasts, there is a significant increase in GAG release from cartilage with live chondrocytes, but no significant increase in GAG release from cartilage from cartilage with dead chondrocytes as compared to the negative control C-O (both dead and live chondrocytes). Furthermore, there was significantly greater GAG release from cartilage explants with live chondrocytes co-cultured with pre-treated osteoblasts as compared to cartilage explants with dead chondrocytes in the same culture conditions. From this we can conclude that the GAG release from the cartilage matrix seen in response to co-culture with pre-treated osteoblasts is an effect of soluble factors produced by osteoblasts, which then alter the phenotype of the chondrocyte (as shown previously with alterations in gene expression of various proteolytic genes in the chondrocyte). It is the live chondrocyte therefore which produces the proteolytic enzymes responsible for cartilage matrix breakdown. The effect of osteoblasts producing soluble factors which are transported via the culture medium to have a direct effect on breakdown of the cartilage matrix would appear to be minimal.

References

Jasin, H.E. and Dingle, J.T. (1981) Human mononuclear cell factors mediate cartilage matrix degradation through chondrocyte activation. *The Journal of clinical investigation* **68**, 571-581.

Metabolomic Profiles of Media from Osteoblasts treated with IL1β and from Co-Culture Systems

4.1 Introduction

While the roots of metabolomics may be traced back to ancient Greece, where the basic idea that changes in tissues and biological fluids are indicative of disease originated (Nicholson and Lindon 2008), the modern techniques of analysis of samples used in metabolomics are relatively new. In the post-genomic era, it has become clear that solely mapping the genes, mRNA and proteins of a living system does not reveal its phenotype. Consequently, researchers have turned their interest to the metabolome (or the metabolic complement of functional genomics) and thus metabolomics is a rapidly expanding post-genomic science that utilises analytical techniques to measure low molecular weight metabolites in biological samples (Dunn *et al.* 2005; Griffin 2003; Wilson *et al.* 2005). The low molecular weight metabolites represent the end products of cell regulatory processes and as such indicate both normal phenotype and pathology. Furthermore, low molecular weight metabolites offer the possibility of identifying biomarkers of disease states due to the potential for abnormal cellular processes to lead to disturbances in the profile of metabolite profiles (Whitfield *et al.* 2004).

The distinction between metabolomics and metabonomics has been described as being mainly philosophical rather than technical (Nicholson and Lindon 2008). Metabonomics is defined as the quantative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification (Nicholson *et al.* 1999). Metabolomics, on the other hand, is defined as the comprehensive analysis of all the low molecular weight metabolites within, or that can be secreted by, a given cell type or tissue under a given set of conditions (Goodacre *et al.* 2004; Nicholson and Wilson 2003). In practice, there is a great deal of overlap between the two techniques with the terms often being used interchangeably and the analytical and modelling procedures used being identical. Full definitions of terms used in metabolomics and related disciplines are as listed in Table 4.1 (Ellis *et al.* 2007).

Term	Definition
Metabolomics	The nonbiased identification and quantification of all metabolites in a biological system
Metabonomics	The quantitative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification
Metabolome	The complete set of low molecular weight metabolites within, or that can be secreted by, a given cell type or tissue
Metabolic Profiling	Identification and quantification of a selective number of predefined metabolites which are generally related to a specific metabolic pathway
Metabolic Fingerprinting	Global, high-throughput analysis to provide sample classification

Table 4.1: Terminology of Metabolomics (Ellis et al. 2007)

The experimental methods employed in metabolomics studies in general involve several stages including sample collection, sample processing, metabolite detection, data analysis and interpretation (Figure 4.1).

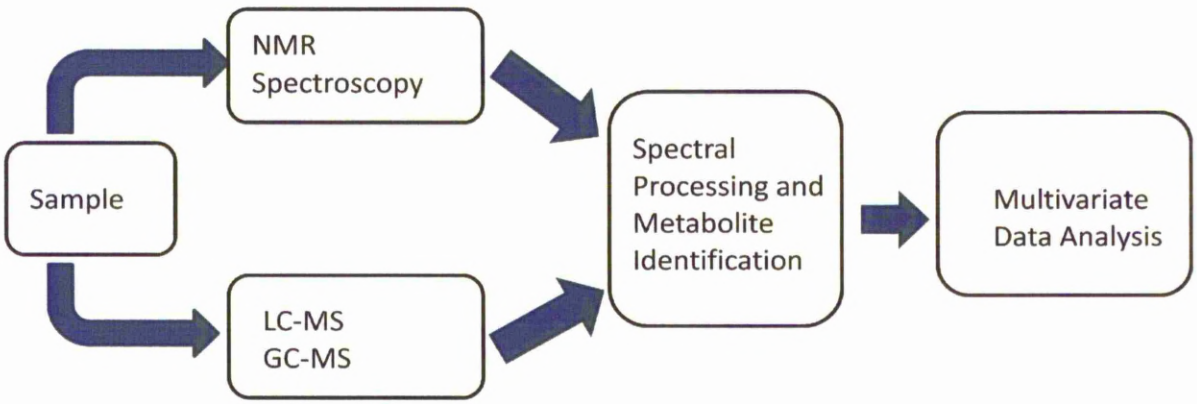

Figure 4.1: Stages of experimental methods in metabolomics. (NMR = nuclear magnetic resonance; LC-MS = liquid chromatography mass spectrometry; GC-MS = gas chromatography mass spectrometry).

The principal analytical techniques used in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Both techniques can generate large amounts of data and complex spectral profiles, which must then be analysed using advanced bioinformatic and statistical methods. Samples of interest are compared with those of controls, such that the spectral features

caused by the disease state can be determined (Nicholson and Lindon 2008). Both NMR and MS platforms have advantages and disadvantages, and likewise their proponents and opponents. As compared to MS, NMR spectroscopy is non-destructive and requires little or no sample preparation, and is therefore capable of generating a comprehensive profile of low-molecular weight metabolites from intact biofluids and tissues (Reo 2002). However, in certain circumstances the ^{1}H NMR spectrum is insufficient to provide information that will fully characterise a metabolite e.g. where analytes contain functional groups that are deficient in protons or where protons can readily chemically exchange with the solvent (Villas-Boas *et al.* 2005). MS analysis therefore, has its most important advantage over NMR in its considerably higher sensitivity.

MS may be combined with separation techniques (e.g. chromatography) to reduce the number of competitive analytes entering the mass spectrometer and to separate complex mixtures of metabolites. This therefore expands the capability of chemical analysis of highly complex biological samples (Pham-Tuan *et al.* 2003; Villas-Boas *et al.* 2005). Both liquid and gas chromatography may be utilised as separation techniques prior to MS. Early technical problems associated with introduction of liquids to a high vacuum system as required in coupling liquid chromatography (LC) with MS have been overcome by the introduction of technical solutions such as soft-ionisation techniques and by the modification of LC methods (Abian 1999; Niessen 1999; Villas-Boas *et al.* 2005). LC-MS has advantages in that this platform has the ability to determine selected metabolites quickly and with simpler sample preparation as compared to GC-MS (Villas-Boas *et al.* 2005).

A limitation of gas chromatography (GC)-MS is that samples must be volatile to be separated on a GC column. Most naturally occurring metabolites are not sufficiently volatile to be analysed directly on a GC system and therefore derivatisation is required, thus adding time and increased variance to the analysis (Villas-Boas *et al.* 2005). Silylation of organic compounds is the classical and most widely used derivatisation procedure for metabolome analysis by GC-MS. A silyl group is introduced into the molecule, in most cases by the substitution of active hydrogens. Disadvantages of silylation

include that reactions must be performed under anhydrous conditions, samples must be heated for over 1 hour, resulting in the loss of thermo-labile metabolites and as the residual reagents in samples after derivatisation are not removed, a resultant decreased column lifetime (Villas-Boas *et al.* 2005).

Large and increasingly complex data sets are generated from metabolomics analysis and subsequently advanced statistical analysis is necessary to maximise interpretation of data. Statistical analysis may be performed using either supervised or unsupervised methods. In unsupervised methods, patterns are determined without prior knowledge of which group the samples are members of. In supervised methods, additional information is added, such as group or clinical data, and similarities and differences between groups are determined (Holmes and Antti 2002). A widely used unsupervised method is principal component analysis (PCA). PCA is recommended as a starting point for analysing multivariate data, rapidly provides an overview of information hidden in the data and can be used to investigate clustering tendency, detect outliers and to visualise data structure (Lu *et al.* 2008). PCA is often followed by a supervised analysis technique such as partial least squares discriminant analysis (PLS-DA). PLS allows analysis of multivariate data where a quantitative relationship between a descriptor matrix X and a response matrix Y is sought. PLS-DA is performed to enhance the separation between groups of observations (Lu *et al.* 2008). Orthogonal projections to latent structures (O-PLS) is a further development of PLS analysis. The objective of this modification is to improve interpretation of PLS models and reduce model complexity (Trygg and Wold 2002).

In mammalian systems, metabolomics has been utilised in the analysis of a wide range of biofluids and tissues in mammalian tissues including cerebrospinal fluid, amniotic fluid, synovial fluid, seminal fluids, bile, saliva and faeces, tumour biopsies and body tissues. Plasma and urine however are the most commonly used materials for studies in humans and animals as they are readily accessible. More specifically, in the field of osteoarthritis a urinary metabolite profile has been identified in the

guinea pig spontaneous OA model (Lamers *et al.* 2005). Metabolomic analysis of cell culture media from explant culture has been previously described for placental tissue (Heazell *et al.* 2008), but to our knowledge has not previously been described in cartilage explant or cartilage explant and osteoblast co-culture systems.

The aim of the experiments described in the following chapter was to investigate the metabolic profile of the cell culture media collected from the *in vitro* experiments using equine osteoblasts stimulated with interleukin 1β (Chapter 2) and from the equine osteoblast and chondrocyte co-culture experiments (Chapter 3). Specific aims were 1) to determine whether metabolites could be reproducibly detected in samples of conditioned media from equine osteoblast and osteoblast and chondrocyte co-cultures and 2) to determine whether, after processing of the spectra, metabolite identification could be utilised to reveal novel biomarkers.

It was hypothesised that there would be detectable differences in the metabolic profiles of conditioned culture media from various equine osteoblast and equine osteoblast and chondrocyte co-culture models, and that useful biomarkers could be identified after analysis of the spectra.

4.2 Materials and Methods

4.2.1 Sample collection

4.2.1.1 Osteoblasts

Equine osteoblasts in monolayer were grown from subchondral bone explants and differentiated as described in Chapter 2 (Culture of Equine Osteoblasts and Response to Interleukin 1β). After differentiation, cells were cultured for 24 hours in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+, with or without the addition of 10ng/mL interleukin 1β (IL1β). Subsequently, cells were washed before culturing in DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+, for a further 72 hours. At the end of the culture period, 1mL

media was collected from the cultures. Experiments were performed in triplicate and repeated with material obtained from 4 separate donors. Samples of the cell culture medium used in each experiment were also collected in triplicate and used as a negative control.

4.2.1.2 Osteoblast and Cartilage Explant Co-culture

Equine osteoblast and cartilage explant co-culture systems were set up as described in Chapter 3 (Co-Culture System of Equine Osteoblasts and Chondrocytes). Briefly, osteoblasts were cultured from explants, differentiated, washed and cultured with or without the addition of 10ng/mL IL1β to the culture media for 24 hours. Subsequently, allogeneic cartilage explants were placed in co-culture inserts with a pore size of 1μm and co-cultures were carried out for 72 hours, with 1mL media being collected from each culture well at the end of the culture period. As controls, samples of medium were also collected from cartilage explants cultured without osteoblasts and from culture medium not cultured with any cells. Medium used for co-culture experiments was DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 500ng/ml amphotericin B and 1% ITS+. Experiments were performed in triplicate and repeated with material obtained from 4 separate donors.

After harvesting, media was immediately frozen in liquid nitrogen and stored at -80°C. Samples were thawed before further extraction and analysis and utilised after one freeze-thaw cycle.

4.2.2 Preparation of Conditioned Culture Medium for GC-MS

Preparation and analysis of metabolic samples were performed in collaboration with Jennifer Kirwan, Faculty of Veterinary Science, University of Liverpool. Unless stated otherwise, all reagents were supplied by Sigma-Aldrich (Dorset, England). Conditioned culture medium was prepared for gas chromatography -mass spectrometry (GC- MS) analysis according to the protocol of Heazell et al. (2008). Two hundred μL of cell-free supernatant of conditioned culture medium was spiked with

100µL 0.19mg/mL succinic d_4 acid as a time-base calibrant. Samples were dried using a vacuum centrifuge (Jouan RC10.22) attached to a cold trap (Jouan RCT 90). Samples were chemically derivatised in a two-stage procedure. Fifty µL *O*-methylhyroxylamine hydrochloride in pyridine (20mg/mL) was added to the samples and incubated at 37°C for 90 minutes. Following this incubation, 50µL MSTFA (*N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide) was added and heated at 37°C for 90 minutes. Following incubation, the derivatised solution was centrifuged at 15339 *x g* for 10 minutes and the supernatant was transferred to a glass vial. Samples were diluted 1:2 with hexane prior to GC-MS analysis. To monitor variation between batches, quality control samples (QC) consisting of a mixture of media from a random selection of samples were derivatised as above and included in the analysis. All samples were injected in duplicate.

4.2.3 GC-MS Analysis

All samples were analysed in a random order. The 1.0mL derivatised sample was injected with a 20:1 split into an Agilent 6980 GC system, which was equipped with an Agilent DB5-MS column (30m x 180µm x 0.18µm) (Stockport, UK). The inlet temperature was set at 250°C. Helium was used as the carrier gas, at a constant flow rate of 1.0mL/min. To achieve good separation, the column temperature was held for 85°C for 2 minutes and then ramped at 15°C/minute to a final temperature of 300°C that was maintained for 2 minutes. The column effluent was introduced into the ion source of a Waters GCT premier mass spectrometer (Waters, Manchester, UK). The transfer line temperature was set at 250°C and the ion source temperature at 180°C. Ions were generated by a 70eV electron beam at a current of 3.2mA. The instrument was calibrated with heptacosa (perfluorotributylamine) reference. The heptacosa was also introduced as the lock mass (*m/z* 218.9856) from the reference reservoir. After a solvent delay of 7 minutes mass spectra were acquired at a rate of $20s^{-1}$ over a mass range of *m/z* 50 to 650.

4.2.4 Data Processing and Analysis

Data were collected using MassLynx applications Manager Software v4.1 (Waters). Compounds were identified by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST) 2005 mass spectral library. Metabolites with a similarity index more than 70% were assigned an identity. The raw GC-MS data were deconvoluted and aligned within mass and retention time windows using the MarkerLynx (version 4.1) applications manager software (Waters) to generate a matrix of *m/z* and retention pairs with associated intensities. Chromatographic peaks in the raw data files were detected by extracting nominal mass chromatograms and tracking the apex of the peaks in the chromatograms. The spectra from each of the detected peaks were saved as retention time and exact mass pairs along with associated intensities. These data were then exported to SIMCA-P+ v11.5 (Umetrics, Sweden) for multivariate data analysis

4.2.5 Multivariate Data Analysis

Principal component analysis (PCA) using Pareto scaling was performed within MarkerLynx applications manager. In Pareto scaling, each variable is divided by the square root of the standard deviation with the goal of reducing the relative importance of large values while keeping the data structure partially intact (van den Berg *et al.* 2006). PCA scores plots produced in MarkerLynx demonstrated any groups formed by clustering of the data. A supervised statistical approach, partial least squares-discriminant analysis (PLS-DA) was performed by exporting the processed data to SIMCA-P+ v11.5 multivariate analysis software package (Umetrics, Sweden). SIMCA-P+ was also used to perform another supervised statistical approach, orthogonal projections to latent structures (OPLS). OPLS scores plots demonstrated any clustering of the data and OPLS loadings plots indicated which ions had the largest influence on the observed variance in the data, i.e. the variables at the top and the bottom of the loadings plot are those that change most, while the ones in the centre do

not vary significantly. Based on mass to charge ratio (*m/z*) ions were identified from chromatographic peaks using the NIST 2008 spectral library. To validate the OPLS model, a training set was created by excluding 6 observations and their repeats at random, then using the remaining samples to create a new OPLS model. The 6 samples were then re-inserted as a test set to test the robustness of the OPLS model. Sensitivity (defined as proportion of true positives correctly identified) and specificity (defined as proportion of true negatives correctly identified) of the model were then calculated from this test set.

Experiment 1 (stimulation of equine osteoblasts grown in monolayer with IL1β) and Experiment 2 (co-culture of equine chondrocytes with equine osteoblasts not pre-treated or pre-treated with IL1β) were performed at separate time points, using different donors and media from different batches. Therefore, data were not pooled and data from Experiments 1 and 2 were analysed and presented separately.

4.3 Results

4.3.1 Qualitative Results

Typical GC-MS total ion chromatograms from conditioned media from osteoblasts and osteoblasts stimulated with IL1β (Experiment 1) are shown in Figure 2. Visual examination of the traces revealed readily identifiable differences in the components of the two groups of media. Identifications of major peaks as numbered in Figure 2 are listed in Table 4.2.

Peak Number	RT (min)	Description from best fit library
1	5.68	L-alanine
2	6.05	Ethanedioic acid (oxalic acid)
3	6.8	L-valine
4	7.34	L-leucine
5	7.56	L-isoleucine
6	7.69	Ethanedoic acid (oxalic acid)
7	8.45	L-threonine
8	9.58	L-proline
9	9.92	α-aminoadipic acid
10	10.48	L-phenylalanine
11	12.60	D-glucose
12	13.02	D-glucose

Table 4.2: Identification of major peaks on chromatograms from Experiment 1 (osteoblasts +/- IL1β) as labelled in Figure 2. (RT= retention time)

Typical GC-MS total ion chromatograms from conditioned media from osteoblasts co-cultured with chondrocytes; osteoblasts pre-treated with IL1β and co-cultured with chondrocytes; and chondrocytes cultured alone (Experiment 2) are as shown in Figure 3. As in Experiment 1, visual examination of the traces revealed identifiable differences in the two groups of media. Identifications of the major peaks are as listed in Table 4.3.

There were very few peaks in the traces after retention times of approximately 15 minutes in any of the samples, indicating that there were no lipophilic compounds detectable in the media.

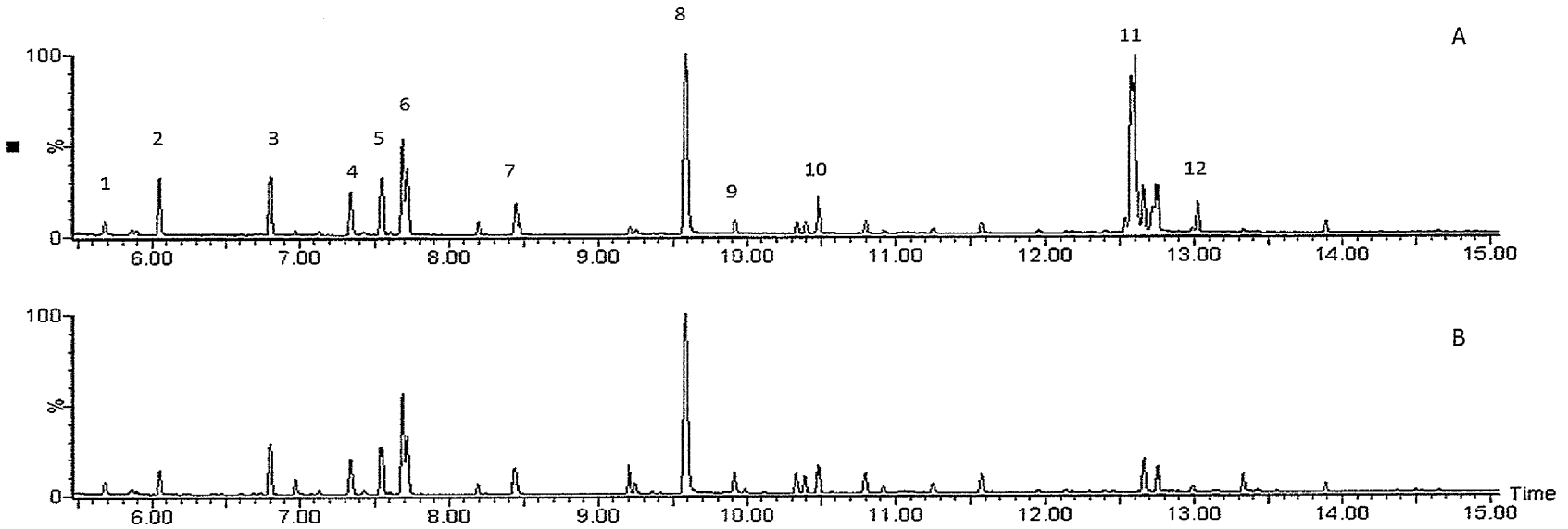

Figure 4.2: Typical total ion chromatograms obtained from GC-MS analysis of (A) conditioned media from osteoblast culture and (B) conditioned media from osteoblasts stimulated with IL1β. Peaks labelled 1-12 are identified in Table 2.

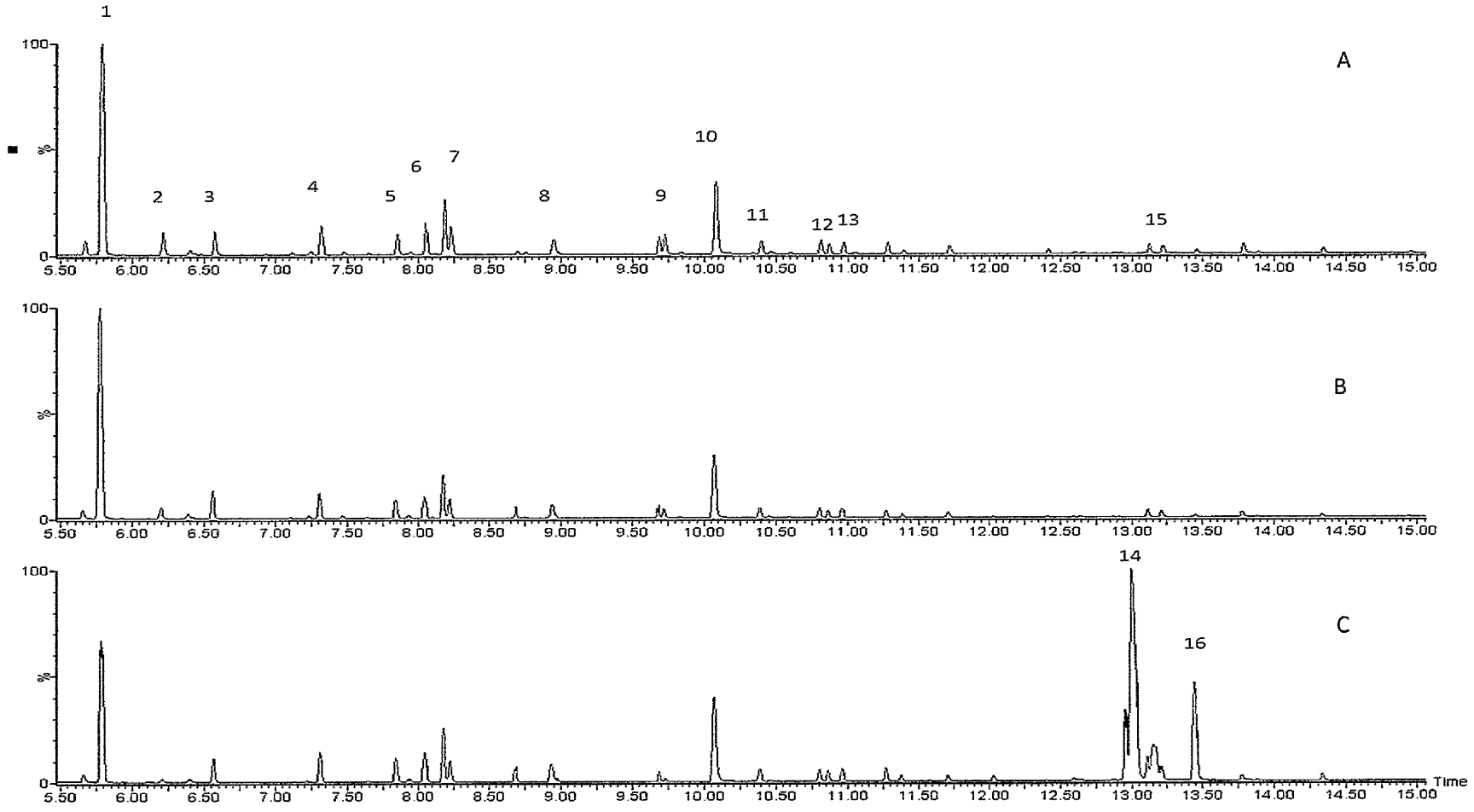

Figure 4.3: Typical total ion chromatograms obtained from GC-MS analysis of (A) conditioned media from osteoblasts co-cultured with chondrocytes, (B) conditioned media from osteoblasts pre-treated with IL1β and co-cultured with chondrocytes and (C) conditioned media from chondrocytes. Peaks labelled 1-16 are identified in Table 3

Peak Number	RT (min)	Description from best fit library
1	5.79	Propanoic acid
2	6.21	L-alanine
3	6.58	Ethanedoic acid (oxalic acid)
4	7.32	L-valine
5	7.85	L-leucine
6	8.05	L-isoleucine
7	8.19	Propanoic acid
8	8.95	L-threonine
9	9.73	L-aspartic acid
10	10.08	L-proline
11	10.40	α-aminoadipic acid
12	10.81	Heptanedioic acid (pimelic acid)
13	10.97	L-phenylalanine
14	13.00	D-glucose
15	13.12	L-lysine
16	13.44	D-glucose

Table 4.3: Identification of major peaks on chromatograms from Experiment 2 (osteoblasts co-cultured with chondrocytes) as labelled in Figure 4.3. (RT= retention time)

4.3.2 Quantitative Results

Further analyses of differences between groups were assessed using statistical methods. Prior to performing statistical analysis using PCA, PLS-DA and OPLS, data was pre-treated using the Pareto scaling method. Scores plots for Experiment 1 are shown for analysis using: PCA (Figure 4.4), PLS-DA (Figure 4.5) and OPLS (Figure 4.6). Scores plots of Experiment 2 are shown for analysis using: PCA (Figure 4.7), PLS-DA (Figure 4.8) and OPLS (Figure 4.9).

On PCA analysis, QC and media negative control samples (Experiment 1) and QC, media negative control and chondrocyte control samples (Experiment 2) were found to be well clustered showing minimal variation during runs and between batches. For further statistical analysis methods (PCA, PL-SDA and OPLS) only the groups of interest involving osteoblast culture were compared, i.e. osteoblasts grown in monolayer and pre-treated or not with IL1β (Experiment 1) or osteoblasts grown in monolayer, pre-treated or not with IL1β then co-cultured with chondrocytes (Experiment 2). Some separation of groups was seen on PCA and good separation of groups was shown with

both PLS-DA and OPLS for Experiment 1. In Experiment 2, separation using the unsupervised technique of PCA was relatively poor but better using the supervised methods. Reasonable separation between groups was shown using PLS-DA for Experiment 2 and more marked separation between groups was found using OPLS.

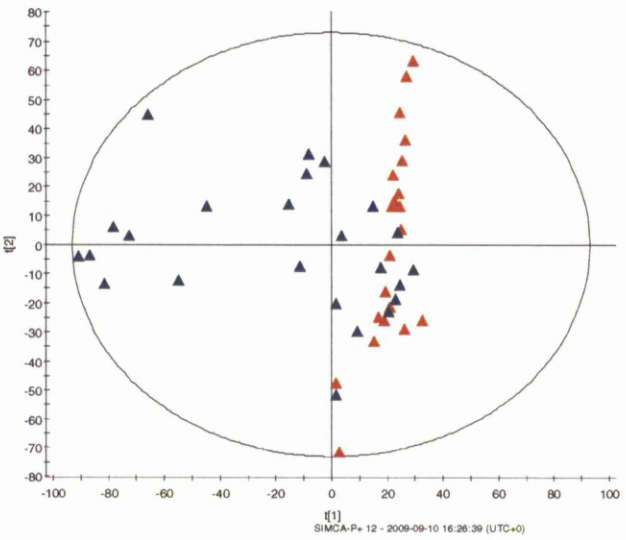

Figure 4.4: Pareto scaled PCA scores plots obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts treated with IL1β (red) and cultures of osteoblasts not treated with IL1β (blue)

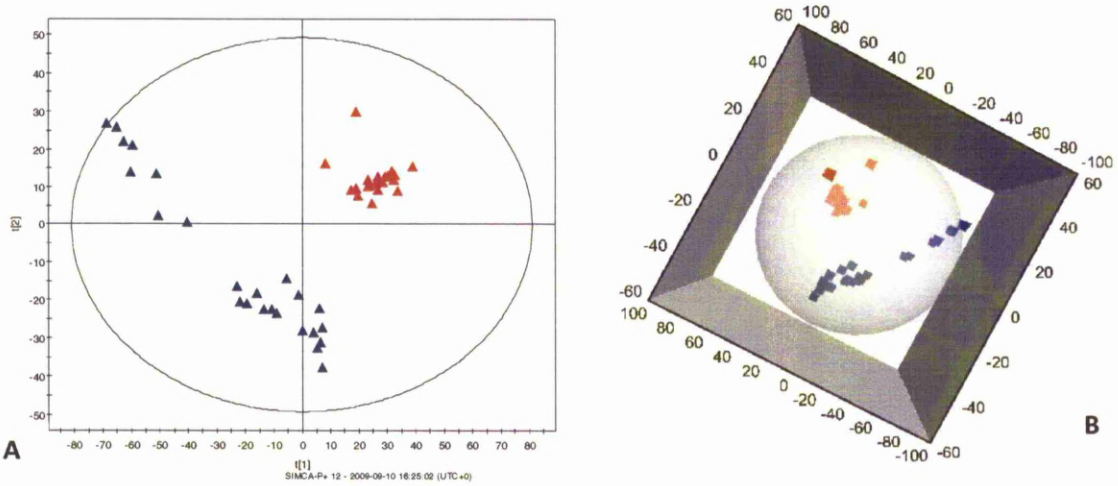

Figure 4.5: (A) 2D model and (B) 3D model of PLS-DA scores plots to 2 components obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts treated with IL1β (red) and cultures of osteoblasts not treated with IL1β (blue)

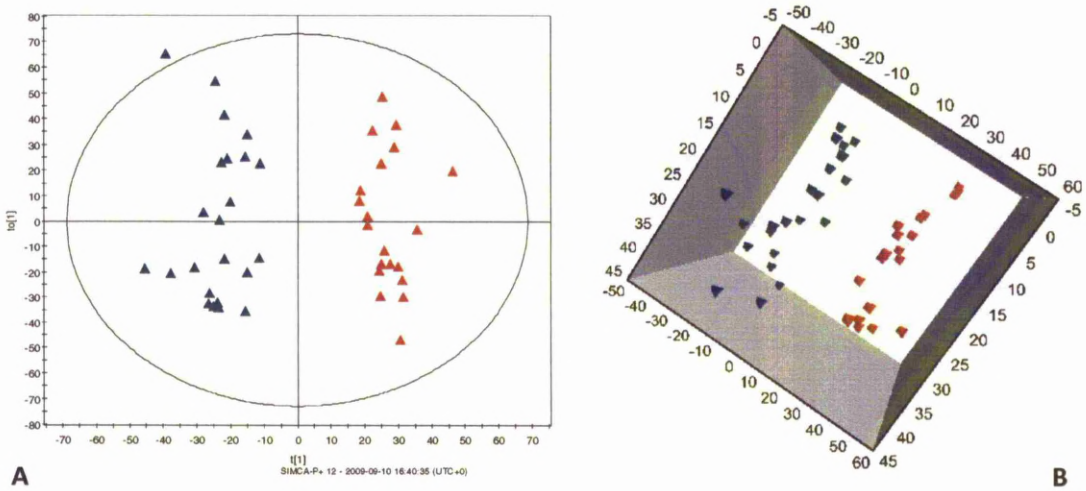

Figure 4.6: (A) 2D model and (B)3D model of Pareto scaled OPLS scores plots obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts treated with IL1β (red) and cultures of osteoblasts not treated with IL1β (blue).

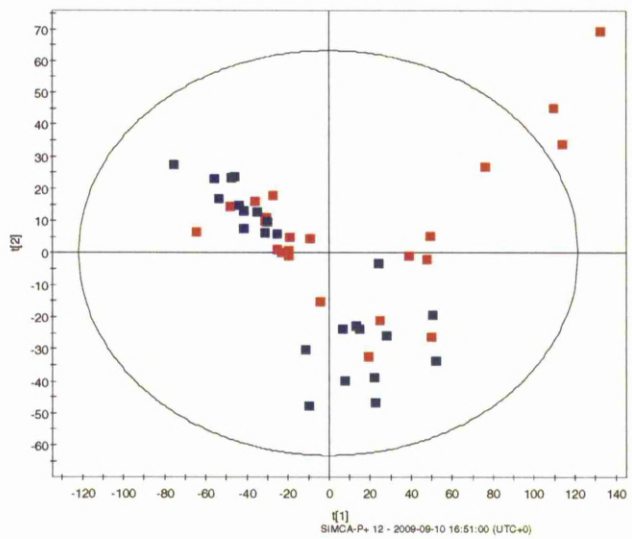

Figure 4.7: Pareto scaled PCA scores plots obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes (red) and cultures of osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes (blue)

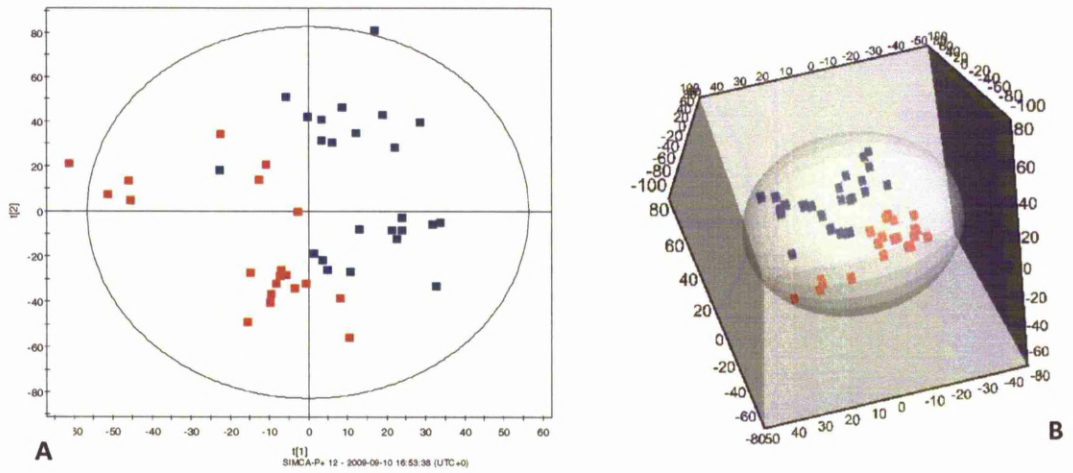

Figure 4.8: (A) 2D model and (B) 3D model of PLS-DA scores plots to 2 components obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes (red squares) and cultures of osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes (blue squares).

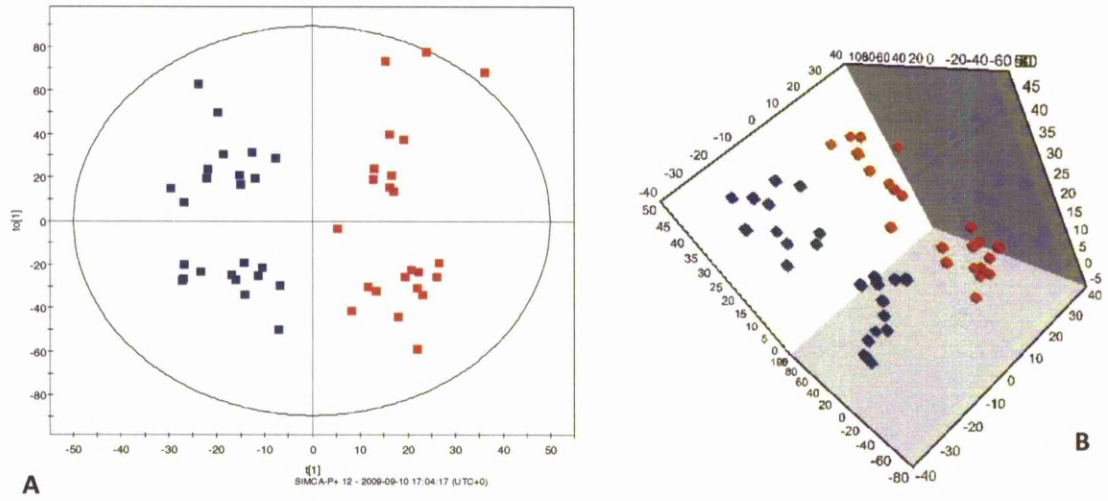

Figure 4.9: (A) 2D model and (B) 3D model of Pareto scaled OPLS scores plots obtained for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes (red squares) and cultures of osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes (blue squares)

OPLS loadings plots are as shown in Figures 4.10 and 4.11 (Experiments 1 and 2 respectively).

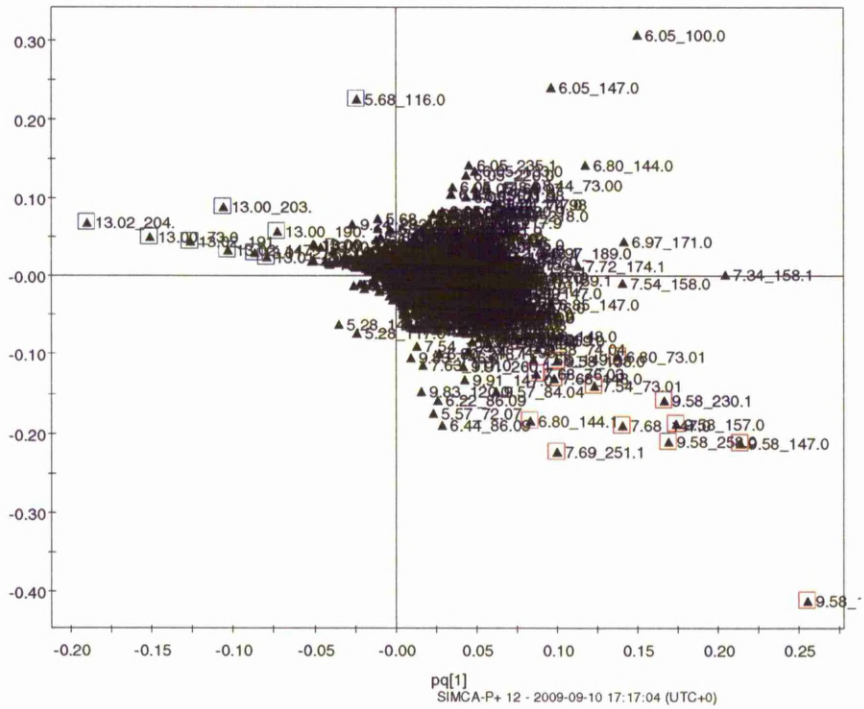

Figure 4.10: Pareto scaled OPLS loadings plot for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts treated and not treated with IL1β. Ions having the largest effect on variance of the data are those at the top and bottom of the plot and are marked with blue and red squares. Numbers represent the retention time and mass to charge ratio (m/z)

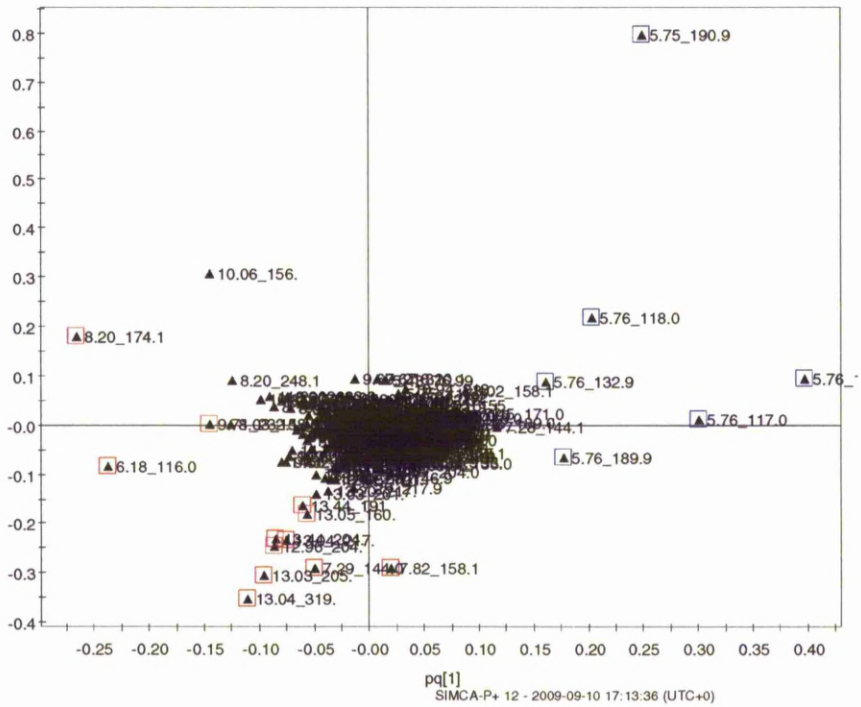

Figure 4.11: Pareto scaled OPLS loadings plot for data derived from GC-MS analysis of media samples obtained from cultures of osteoblasts pre-treated with IL1ß and co-cultured with chondrocytes, and cultures of osteoblasts not pre-treated with IL1ß and co-cultured with chondrocytes. Ions having the largest effect on variance of the data are those at the top and bottom of the plot and are marked with blue and red squares. Numbers represent the retention time and mass to charge ratio (m/z)

Ions having the largest effect on the variance of the data were identified using the NIST 2008 spectral library are shown in full in Tables 4.4-4.7. Briefly, in the monolayer osteoblast experiment, there was increased L-valine, L-isoleucine, glycine and L-proline; and decreased L-alanine and D-glucose in the media of the osteoblasts pre-treated with IL1β. In the co-culture experiment there was increased propanoic acid; and decreased L-alanine, L-valine, L-leucine, glycine, L-aspartic acid and D-glucose in the media of the osteoblasts pre-treated with IL1β and co-cultured with chondrocytes.

RT (min)	Principal ions observed (*m/z*)	Elemental composition	MW	Description from best library fit
6.80	144, 73	$C_{11}H_{27}NO_2Si_2$	261	L-Valine
7.54	73	$C_{12}H_{29}NO_2Si_2$	275	L-Isoleucine
7.68	147, 148, 76, 251	$C_{11}H_{29}NO_2Si_3$	291	Glycine
9.58	147, 156, 157, 158, 230, 258	$C_{11}H_{23}NO_3Si_2$	273	L-Proline

Table 4.4: Compounds detected by GC/MS as being increased in the media of osteoblasts pre-treated with IL1β as compared to osteoblasts not pre-treated with IL1β. The table shows the principal ions observed and the description from best library fit.
(RT = retention time)

RT (min)	Principal ions observed (*m/z*)	Elemental composition	MW	Description from best library fit
5.68	116	$C_9H_{23}NO_2Si_2$	233	L-Alanine
13.01	73, 191, 204, 217	$C_{21}H_{52}O_6Si_5$	540	D-Glucose

Table 4.5: Compounds detected by GC/MS as being decreased in the media of osteoblasts pre-treated with IL1β as compared to osteoblasts not pre-treated with IL1β. The table shows the principal ions observed and the description from best library fit.
(RT = retention time)

RT (min)	Principal ions observed (*m/z*)	Elemental composition	MW	Description from best library fit
5.76	117, 118, 133, 147, 190, 191	$C_9H_{22}O_3Si_2$	234	Propanoic acid

Table 4.6: Compounds detected by GC/MS as being increased in the media of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes, as compared to osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes. The table shows the principal ions observed and the description from best library fit.
(RT = retention time)

RT (min)	Principal ions observed (*m/z*)	Elemental composition	MW	Description from best library fit
6.18	116	$C_9H_{23}NO_2Si_2$	233	L-Alanine
7.29	144	$C_{11}H_{27}NO_2Si_2$	261	L-Valine
7.82	158	$C_{12}H_{29}NO_2Si_2$	275	L-Leucine
8.20	174	$C_{11}H_{29}NO_2Si_3$	291	Glycine
9.71	232	$C_{13}H_{31}NO_4Si_3$	349	L-Aspartic acid,
12.96	204	$C_{22}H_{55}NO_6Si_5$	569	D-Glucose

Table 4.7: Compounds detected by GC/MS as being decreased in the media of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes, as compared to osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes. The table shows the principal ions observed and the description from best library fit
(RT = retention time)

On validation of the OPLS models by creating training and test sets, it was shown that the sensitivity and specificity of the OPLS models were 71% and 100% respectively for Experiment 1, and 75% and 100% respectively for Experiment 2.

4.4 Discussion

It has been shown that GC-MS is capable of discriminating between samples of media conditioned by 1) culture of equine osteoblasts and equine osteoblasts stimulated by IL1β and 2) co-culture of equine osteoblasts pre-treated or not with IL1β and equine chondrocytes. Previous experiments have shown various *in-vitro* effects of the equine osteoblast and the equine osteoblast stimulated by application of IL1β at gene level and also in terms of MMP-13 protein production and GAG release from the extracellular matrix of cartilage explants (see Chapter 3). It has also been shown that cartilage matrix degradation effects are mediated via the chondrocyte via a soluble factor in the cell culture media (See Chapter 3 Appendix B) and (Jasin and Dingle 1981; Westacott 2002).

Analysis of the GC-MS data derived from the cell media, indicated that there were alterations in various biochemically relevant biomarkers when osteoblasts were pre-treated with IL1β and also when osteoblasts were pre-treated with IL1β and co-cultured with chondrocytes. Interestingly, although there were similarities in the alterations in compounds between experiments, the alterations were not identical.

In both the osteoblast and co-culture experiments, there was decreased D-glucose in the media conditioned by osteoblasts pre-treated with IL1β as compared to osteoblasts not undergoing IL1β treatment. IL1β has been used as a driver of the osteoarthritic phenotype in this sequence of experiments, and its decrease as observed may indicate that in this *in vitro* model of OA there are alterations of glucose metabolism. The decreased glucose in the media may be explained by the equine osteoblasts stimulated with IL1β having increased uptake of glucose, as has been shown

previously to occur in human fibroblasts (Bird *et al.* 1990), synoviocytes (Taylor *et al.* 1988) and chondrocytes (Hernvann *et al.* 1996).

As well as alterations in glucose, changes in amino-acid content of the cell culture medium were also observed. Although alterations in amino acid utilisation and formation have not been shown in osteoblasts in cell culture to our knowledge, previously it has been shown that chicken growth plate epiphyseal chondrocytes in short term culture release alanine, glycine, proline and aspartate into the cell culture medium (Ishikawa *et al.* 1985). Our results add further information to these previous findings. In both the osteoblast and the co-culture experiments, there was decreased alanine in the medium of the cultures where the osteoblasts had been driven towards an osteoarthritic phenotype via pre-treatment with IL1β. Alanine is a non-essential amino acid which is not routinely present in DMEM culture medium. Therefore the reduction of the compound identified in the culture medium may indicate a true reduction in synthesis by the osteoblasts pre-treated with IL1β in both monolayer and under co-culture with chondrocytes conditions.

The response of valine and glycine is interesting in that these amino acids were increased in the media of osteoblasts pre-treated with IL1β as compared to osteoblasts not pre-treated with IL1β, but in the co-culture experiment these same amino acids were decreased in the media of osteoblasts pre-treated with IL1β and co-cultured with chondrocytes as compared to osteoblasts not pre-treated with IL1β and co-cultured with chondrocytes. Valine is an essential amino acid which therefore must be added to cell culture media for cell survival (Eagle 1959) and is included in commercially available DMEM. Although glycine is not an essential amino acid, it is also routinely added to commercially available DMEM. The relative increase and decrease in the cell culture medium of these amino acids may represent a reduced and increased utilisation respectively by the cells in the various culture conditions. It is suggested that the difference between experiments may be associated with co-culture with chondrocytes as this was only difference in the experimental design. Potentially, although utilisation is decreased in the IL1β pre-treated osteoblasts, when these

IL1β driven osteoblasts are co-cultured with chondrocytes, the phenotype of the chondrocyte is altered such that valine and glycine utilisation are increased, resulting in a relative decrease in these amino acids in the culture medium. Conversely, another explanation for the reduced glycine content of the media in the co-cultures of IL1β pre-treated osteoblasts and chondrocytes may be a reduction in synthesis by the chondrocytes. This is of particular interest in this *in vitro* model of osteoarthritis as collagen is composed of approximately 35% glycine (Nelson and Cox 2005).

4.5 Conclusion

In conclusion, the technique of GC-MS has been shown to be capable of allowing identification of detectable differences in the metabolic profiles of conditioned culture media. Biologically relevant markers in the culture media of this model of osteoblast driven osteoarthritis have been identified. Cell metabolism has been altered as observed via changes in both glucose and glucose metabolism. Alterations in compounds in the cell culture media were different in the osteoblast +/- IL1β pre-treatment experiment as compared to the osteoblast +/- IL1β pre-treatment co-culture with chondrocytes experiment, indicating an effect of co-culture with chondrocytes.

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Post Mortem Evaluation of Palmar Osteochondral Disease (Traumatic Osteochondrosis) of the Metacarpo-/Metatarsophalangeal Joint in Thoroughbred Racehorses

5.1 Introduction

The condition which we refer to as palmar/plantar osteochondral disease (POD) of the third metacarpal and metatarsal (MC/MTIII) condyles has previously been referred to as traumatic osteochondrosis (Pool 1996) in the equine veterinary literature. Although initially considered to be a manifestation of osteochondritis dissecans (Hornof et al. 1981), this condition is now believed to be a biomechanical disorder, resulting from repetitive overload trauma in horses undergoing cyclic high intensity exercise (Pool 1996).

The clinical condition of pain associated with subchondral bone injury of the distal condyles of MC/MTIII is recognised in most racing breeds including Thoroughbreds (Arthur et al. 2003; Pilsworth 2003), Standardbreds (Mitchell et al. 2003; Torre 2003), Quarter Horses (Lewis 2003) and Scandinavian Cold-Blooded Trotters (Ertola and Houttu 2003). Grossly the lesions are characterised as small, ovoid defects in the palmar or plantar articular surface of the condyle of approximate 2-4mm diameter (Pool 1996). Lesions are centred about 5-8mm proximal to the transverse ridge and may be located 3-15mm from the sagittal ridge on either the medial or lateral condylar surfaces (Pool 1996). Lesions appear to vary in severity. Early findings include a focus of bluish discolouration of the subchondral bone visible through grossly normal articular cartilage. More severe changes include physical disruption of the subchondral bone associated with varying degrees of pathology of the overlying articular cartilage. Ultimately there may be collapse of the subchondral bone with ulceration of the articular cartilage (Riggs 2006). In some cases these lesions have been reported to be associated with catastrophic condylar fracture. (Krook and Maylin 1988).

The pathological changes are clinically evident as a performance limiting lameness which may be bilateral or, in some cases, quadrilateral and as a result the affected horse may present with a poor action rather than overt lameness (Pilsworth 2003). The condition may be difficult to diagnose due to an inconsistent response to intra-articular analgesia and because only severe lesions are appreciable on routine radiography of the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints (Richardson 2003). More specialised radiographic views may aid diagnosis (O'Brien et al. 1981; Pilsworth et al. 1988) and specialised imaging modalities such as nuclear scintigraphy (Ross 1998; Richardson 2003), computed tomography (Byron and Goetze 2007; Morgan et al. 2006) and magnetic resonance imaging (Zubrod et al. 2004) are extremely useful in diagnosis of POD. Visualization of the predilection site for POD lesions on the palmar/plantar aspect of the condyles of MC/MTIII is physically impossible during routine arthroscopic examination of the MCP/MTP joints. Consequently, many lesions remain undiagnosed until the later stages of disease, by which time the changes are irreversible and will result in chronic joint disease.

The aims of the study were to 1) to evaluate gross pathology of the distal condyles of MC/MTIII of Thoroughbred racehorses at post-mortem examination and develop a scoring system to document relevant findings, 2) to describe the prevalence and distribution of POD lesions within a population of flat racing Thoroughbreds and 3) to determine any relationship between pathologies of the condyles of MC/MTIII (wear lines, cartilage loss/ulceration, marginal remodelling, linear fissures and dorsal impact injuries) and POD. It was hypothesised that: 1) POD will have a high prevalence in a population of intensively raced Thoroughbreds, 2) There will be predilection sites for these pathologies, 3) there will be an association between POD and other pathologies affecting the distal condyles of MC/MTIII and 4) There will be a correlation between prevalence of POD and pathologies of the dorsodistal aspect of MC/MTIII, suggestive of MCP/MTP joint hyperextension.

5.2 Materials and Methods

Material was examined from 64 Thoroughbred racehorses that were in active race training or had been retired from active race training at the Hong Kong Jockey Club and required euthanasia over a 15 month period. Material collection and gross *post-mortem* grading were performed by collaborators at the Hong Kong Jockey Club (Dr Christopher Riggs, Peter Curl and Suzanne Troester). Forty nine/64 horses were euthanased as a result of orthopaedic disease (fracture n=14; tendon/ligament injury n=12; osteoarthritis n=11; miscellaneous lameness n=11; laminitis n=1). The remainder of horses (13/64) were euthanased for reasons unrelated to orthopaedic disease.

The distal articular surface of MC/MTIII was examined by gross observation at post mortem examination performed immediately after death by one observer (CMR). A scoring system was developed and each condyle was assigned a score for POD, wear lines, cartilage loss, marginal remodelling, linear fissures and dorsal impact injuries (Table 5.1). Examples of affected condyles and their scoring are as shown in Figures 5.1 a-d.

Scores were entered into an Excel for Microsoft Office 2003 (Microsoft Corp, Redmond, USA) database and descriptive data obtained. The statistical significance of distribution of lesions and POD was determined using the Chi-square test of association. The statistical significance of the relationships between grades of pathologies at the condylar level was determined using the Chi - squared test, the Gamma statistic for ordinal data and ordinal logistic regression. SPSS for Windows 14.0 (SPSS Inc, Chicago, USA) and Minitab for Windows 14.0 (Minitab Inc, Pennsylvania, USA) were used for statistical analysis.

Feature	Score	Description
Palmar/Plantar osteochondral disease	0	No evidence of POD
	1	Discolouration (bruising) of subchondral bone only, no or minimal disruption of overlying articular cartilage
	2	Discolouration (bruising) with mild to moderate disruption of articular cartilage
	3	Established POD lesions. Discolouration and disruption/collapse of articular surface
Wear lines in cartilage	0	Wear lines absent
	1	Partial thickness wear lines in cartilage
	2	Full thickness wear lines in cartilage
Cartilage loss – associated with transverse ridge, condylar groove and central condylar region	0	No evidence of cartilage loss
	1	Partial thickness cartilage loss (fibrillation)
	2	Full thickness cartilage loss (ulceration) with minor exposure of calcified cartilage/subchondral bone
	3	Extensive full thickness cartilage loss with exposure of the subchondral bone over an area >5mm in diameter
Linear fissures (within condylar grooves)	0	No evidence of linear fissures
	1	Faint groove with intact cartilage visible along length
	2	Well defined groove with partial thickness split in cartilage
	3	Well defined groove with full-thickness split in cartilage
Marginal remodelling	0	Marginal remodelling absent
	1	Marginal remodelling present
Dorsal impact injury	0	No evidence of dorsal impact injury
	1	Mild (thickening/inflammation of synovial pad and mild erosion of underlying MC/MTIII, mild erosion dorsoproximal P1)
	2	Severe (thickened, inflamed synovial pad and marked erosion MC/MTIII, full thickness cartilage loss and/or chip fracture of dorsoproximal P1)

Table 5.1: Scoring system for metacarpo/metatarsophalangeal pathology

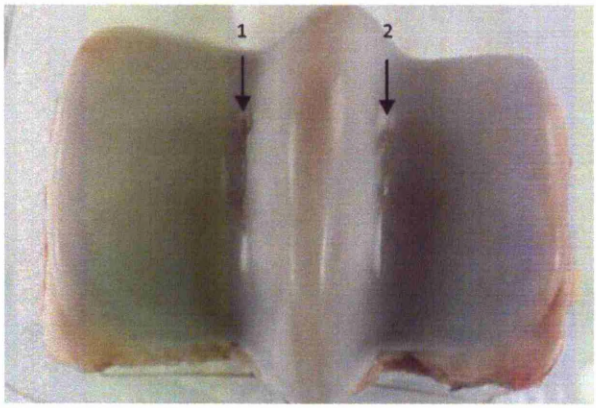

Figure 5.1a: Example of the plantar aspect of a distal metatarsus (lateral to the right) from a 6 year old Thoroughbred that had 32 race starts during its career. This was graded as having grade 0 palmar ostechondral disease (both condyles), Grade 0 wear lines (both condyles), Grade 0 cartilage loss (both condyles) and Grade 2 linear fissures (medial condyle, Arrow 1) and Grade 1 linear fissures (lateral condyle, Arrow 2)

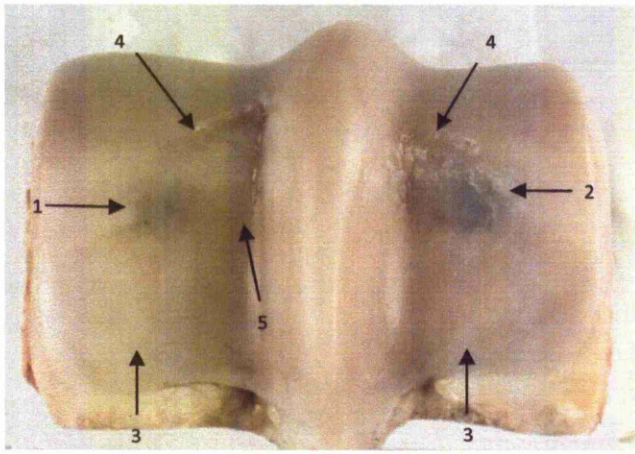

Figure 5.1b: Example of the plantar aspect of a distal metatarsus (lateral to the right) from a 7 year old Thoroughbred that had 45 race starts during its career. This was graded as having Grade 1 palmar ostechondral disease (medial condyle, Arrow 1), Grade 2 palmar osteochondral disease (lateral condyle, Arrow 2), Grade 1 wear lines (both condyles, Arrows 3), Grade 1 cartilage loss (both condyles, Arrows 4), Grade 1 linear fissures (medial condyle, Arrow 5), Grade 0 linear fissure (lateral condyle)

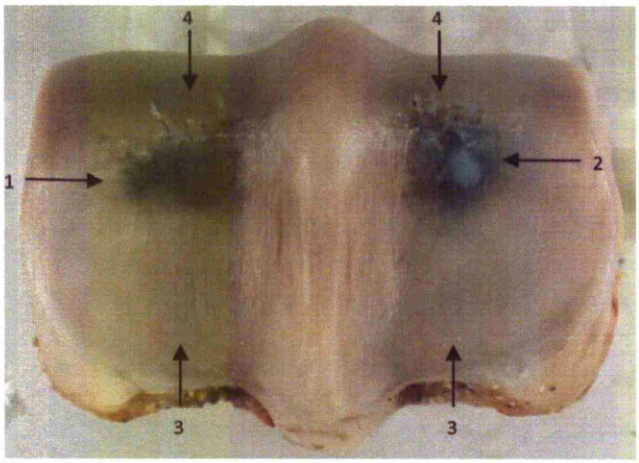

Figure 5.1c: Example of the palmar aspect of a distal metacarpus (lateral to the right) from a 7 year old Thoroughbred that had 45 race starts during its career (the same horse as 1b). This was graded as having Grade 1 palmar ostechondral disease (medial condyle, Arrow 1), Grade 2 palmar osteochondral disease (lateral condyle, Arrow 2), Grade 1 wear lines (both condyles, Arrows 3), Grade 1 cartilage loss (both condyles, Arrows 4) and Grade 0 linear fissures (both condyles)

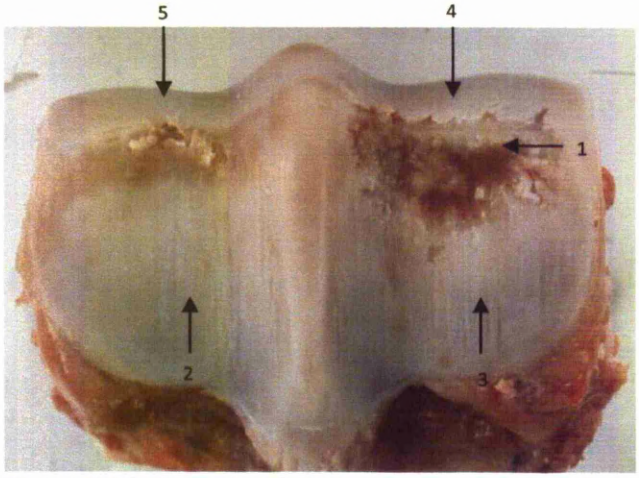

Figure 5.1d: Example of the palmar aspect of a distal metacarpus (lateral to the left) from a 7 year old Thoroughbred that had 48 race starts during its career. This was graded as having Grade 3 palmar ostechondral disease (medial condyle, Arrow 1), Grade 0 palmar osteochondral disease (lateral condyle), Grade 1 wear lines (lateral condyle, Arrow 2), Grade 2 wear lines (medial condyle, Arrow 3), Grade 3 cartilage loss (medial condyle, Arrow 4), Grade 2 cartilage loss (lateral condyle, Arrow 5) and Grade 0 linear fissures (both condyles)

5.3 Results

Sixty four horses were examined. There were 62 geldings and 2 colts. The mean age at retirement was 6.1 years (range 3-10 years) and the mean age at euthanasia was 6.95 years (range 3-18 years). POD was recorded (Grade 1-3 on at least one condyle) in 43/64 of horses, giving a prevalence by individual of 67% (95% CI 54.3, 78.4). A total of 499 of 512

condyles were examined. Two hundred and ninety seven condyles (59.5%) had Grade 0 POD, 118 condyles (23.6%) had Grade 1 POD, 70 condyles (14%) had Grade 2 POD and 14 condyles (2.8%) had Grade 3 POD. It was not possible to accurately score all features of the condyles in every case on account of extensive pathology associated with acute trauma to the joint: condylar fracture (number of horses=2), proximal sesamoid bone fracture (n=2), rupture of the distal sesamoid ligaments (n=1), septic joint (n=1). Data was omitted for the affected limb in each case. In three horses (7 condyles) we found evidence of discrete areas (approximately 2mm in diameter) of opaque but intact cartilage at the site commonly affected by POD lesions. This region of cartilage was smooth and well integrated with the surrounding hyaline cartilage and the underlying subchondral bone appeared grossly normal. Since these could not be graded as normal (POD Grade 0), nor did the pathology fulfil the criteria of POD as the underlying bone was normal, we also omitted them from further analysis in this study.

The distribution of POD lesions was such that POD was present on 98/249 right limb condyles and 104/250 left limb condyles; POD was present on 107/250 forelimb condyles and 95/249 hind limb condyles; POD was present in 93/248 lateral condyles and 109/251 medial condyles. There was no significant correlation between the distribution of POD lesions on left vs right condyles (P=0.6), fore vs hind limb condyles (P=0.3) and lateral vs medial condyles (P=0.2).

When fore and hind limbs were assessed separately, it was found that within the forelimbs 47/125 lateral condyles and 60/125 medial condyles were affected by POD. This gave a trend towards POD lesions occurring more commonly in the medial condyle (P=0.1), but this was not significant. In the hind limbs 46/123 lateral and 49/126 medial condyles were affected by POD. Therefore there was no significant difference between medial and lateral condyles

in the hind limbs (P=0.8). When the effect of left or right limb was assessed separately within the fore and hind limbs, it was found that 52/125 right forelimb condyles and 55/125 left forelimb condyles were affected. Forty six/124 right hind limb condyles were affected and 49/124 left hind limb condyles were affected. Therefore, there was no significant difference in POD lesion distribution between left and right (P=0.7 forelimbs and P=0.7 hind limbs).

The number of condyles affected at horse level and the distribution of the lesions are shown in Table 5.2. Briefly 1 condyle was affected in 7 horses (10.9%), 2 condyles were affected in 6 horses (9.4%), 3 condyles were affected in 6 horses (9.4%), 4 condyles were affected in 3 horses (4.7%), 5 condyles were affected in 1 horse (1.6%), 6 condyles were affected in 8 horses (12.5%), 7 condyles were affected in 4 horses (6.3%) and all 8 condyles were affected in 9 horses (14.1%).

Total number of condyles affected	Condyle								
	Left MCIII medial	Left MCIII lateral	Right MCIII medial	Right MCIII lateral	Left MTIII medial	Left MTIII lateral	Right MTIII medial	Right MTIII lateral	Number of horses (% total)
0	-	-	-	-	-	-	-	-	20 (31.3%)
1	3	1	0	1	0	0	2	0	7 (10.9%)
2	1	2	1	2	2	2	1	1	6 (9.4%)
3	4	1	3	3	1	2	2	2	6 (9.4%)
4	3	2	2	2	2	0	1	0	3 (4.7%)
5	0	0	1	1	1	1	0	1	1 (1.6%)
6	8	4	8	4	7	6	5	6	8 (12.5%)
7	4	4	4	2	4	3	3	4	4 (6.3%)
8	9	9	9	9	9	9	9	9	9 (14.1%)
Number of condyles (% total)	32 (15.8%)	23 (11.4%)	28 (13.9%)	24 (11.9%)	26 (12.9%)	23 (11.4%)	23 (11.4%)	23 (11.4%)	

Table 5.2: Number of condyles affected by palmar osteochondral disease and distribution of lesions
MCIII = third metacarpal; MTIII = third metatarsal

The grades of POD, linear fissures and dorsal impact injuries were recorded in 499 of 512 condyles, wear lines and cartilage loss were recorded in 506 of 512 condyles and marginal remodelling was recorded in 496 of 512 condyles. The numbers of condyles affected by each grade of pathology are summarised in Table 5.3.

	Number of Condyles in Each Grade (% total row)				
Pathology	**Grade 0**	**Grade 1**	**Grade 2**	**Grade 3**	**Total**
Palmar Osteochondral Disease	297 (59.5%)	118 (23.6%)	70 (14%)	14 (2.8%)	499
Wear Lines	158 (31.2%)	246 (48.6%)	102 (20.2%)	-	506
Cartilage Loss	263 (52%)	230 (45.5%)	6 (1.2%)	7 (1.4%)	506
Linear Fissures	248 (49.7%)	189 (37.9%)	62 (12.4%)	-	499
Dorsal Impact Injury	357 (71.5%)	102 (20.4%)	40 (8%)	-	499
Marginal Remodelling	471 (95%)	25 (5%)	-	-	496

Table 5.3: Summary of numbers of condyles affected by each grade of pathology

There was a significant linear relationship (Gamma statistic P-value <0.001) between grade of POD and grades of wear lines, cartilage ulceration and dorsal impact injuries i.e. as the grade of POD increased, the grades of these pathologies increased. There was a significant relationship between grade of POD and grade of linear fissures (Chi-squared P value = 0.008) however this was not linear (GS P value =0.2). There was no relationship between grade of POD and grade of marginal remodelling (GS P value = 0.6; Chi-squared p value = 0.85). This is shown in Table5. 4.

Metacarpo/metatarsophalangeal Pathology			Palmar Osteochondral Disease			
			0	1	2	3
Wear Lines	0	Number of Condyles (column %)	128 (43.2%)	20 (16.9%)	10 (14.3%)	0 (0%)
	1	Number of Condyles	114 (38.5%)	84 (71.2%)	40 (57.1%)	7 (50%)
	2	Number of Condyles	54 (18.2%)	14 (11.9%)	20 (28.6%)	7 (50%)
Total		Number of Condyles	296	118	70	14
Chi-Square P Value GS P Value Wear Lines vs POD			<0.001 <0.001			
Ulceration	0	Number of Condyles	180 (60.8%)	58 (49.2%)	19 (27.1%)	5 (35.7%)
	1	Number of Condyles	112 (37.8%)	58 (49.2%)	49 (70.0%)	6 (42.9%)
	2	Number of Condyles	2 (0.7%)	2 (1.7%)	1 (1.4%)	0 (0%)
	3	Number of Condyles	2 (0.7%)	0 (0%)	1 (1.4%)	3 (21.4%)
Total		Number of Condyles	296	118	70	14
Chi-Square P Value GS P Value Ulceration vs POD			<0.001 <0.001			
Linear Fissures	0	Number of Condyles	139 (46.8%)	49 (43.8%)	43 (62.3%)	11 (84.6%)
	1	Number of Condyles	125 (42.1%)	45 (40.2%)	16 (23.2%)	1 (7.7%)
	2	Number of Condyles	33 (11.1%)	18 (16.1%)	10 (14.5%)	1 (7.7%)
Total		Number of Condyles	297	112	69	13
Chi-Square P Value GS P Value Linear Fissures vs POD			=0.008 =0.2			
Marginal Remodelling	0	Number of Condyles	280 (94.9%)	105 (95.5%)	62 (95.4%)	14 100.0%
	1	Number of Condyles	15 (5.1%)	5 (4.5%)	3 (4.6%)	0 (0%)
Total		Number of Condyles	295	110	65	14
Chi-Square P Value GS P Value Marginal Remodelling vs POD			=0.85 =0.6			
Dorsal Impact Injuries	0	Number of Condyles	239 (80.7%)	66 (58.4%)	43 (63.2%)	5 (35.7%)
	1	Number of Condyles	44 (14.9%)	33 (29.2%)	16 (23.5%)	6 (42.9%)
	2	Number of Condyles	13 (4.4%)	14 (12.4%)	9 (13.2%)	3 (21.4%)
Total		Number of Condyles	296	113	68	14
Chi-Square P Value GS P Value Dorsal Impact Injury vs POD			<0.001 <0.001			

Table 5.4: Distribution of grades of MC/MTIII condylar pathology with grades of palmar osteochondral disease and statistical significance of the relationships between pathologies
POD = palmar osteochondral disease; GS = Gamma statistic
The column % represents the percentage of each grade of pathology within each grade of palmar osteochondral disease

The relationship between grade of POD and grade of linear fissures was further investigated using ordinal logistic regression. It was found that condyles with Grade 1 linear fissures were significantly more likely to have lower grades of POD than either condyles with Grade 0 or Grade 2 linear fissures (P=0.007). This is shown in Table 5.5.

	Coefficient (s.e.)	Odds ratio (95% CI odds ratio)	P-value
Linear fissures			
0			
1*	0.5 (0.2)	1.7 (1.2, 2.5)	0.007
2	-0.03 (0.3)	1.0 (0.6, 1.6)	0.9

Table 5.5: Ordinal logistic regression model of linear fissures compared to grade of palmar osteochondral disease
The odds ratios presented are from ordinal logistic regression with the lowest POD category being zero; an OR<1 indicates increased probability of being in the higher POD categories; an OR>1 indicates increased probability of being in the lower POD categories.
* A comparison of grade 1 to 2 linear fissures showed that grade 1 linear fissures were also more likely to have lower POD grades than grade 2 linear fissures (OR 1.8 95% CI 1.0,3.11; P-value 0.05)

5.4 Discussion

Palmar osteochondral disease was common in this sample of animals presented for post mortem from a population of racing Thoroughbreds. Whilst 67% of all horses had POD lesions in at least one condyle, most of the identified lesions were not severe (23.6% of affected condyles grade 1, 14% grade 2, 2.8% grade 3). The clinical relevance of these different grades of POD has yet to be established. While it is reasonable to suspect that lesions may progress in severity, this is as yet unproven, as is whether they can resolve.

An interesting and unexpected finding of the study was the discovery of discrete areas of opaque, smooth well integrated cartilage at the site of POD lesions. To our knowledge these

have not been previously described and since we remain unsure of the aetiology of these lesions, these were omitted from analysis. These lesions may be areas of fibrocartilage at the site of a previous POD lesion, or could have some form of developmental aetiology.

No significant difference was found between the distribution of POD lesions on fore vs hind limbs, left vs right limbs or medial vs lateral condyles. This contradicts the previous clinical impressions of Pool (1996) who stated that POD/traumatic osteochondrosis was more common in the forelimbs and that involvement of the medial or lateral condyle appeared to be random. Furthermore, there was no apparent left/right limb bias in either the fore or hind limbs which is surprising given that this population of horses train and race almost exclusively in a clock-wise direction. The association of orthopaedic disease and limb affected/direction of racing has been investigated previously in studies on limb fracture during racing. Our finding of a lack of association between limb affected and direction of racing is supported by Parkin et al. (2006) in work relating to catastrophic distal limb fractures. However earlier descriptive data has suggested that there may be some association between limb injured and racing direction with studies showing a predilection for fracture of the left (inside) forelimb in horses which raced predominantly in an anti-clockwise direction (Rick et al. 1983; Rooney 1983).

As well as showing the high prevalence of POD in this population of racing Thoroughbreds, this study also revealed a high prevalence of wear lines and cartilage loss affecting the distal condyle of MC/MTIII. However, the more severe forms of cartilage loss (i.e. full thickness cartilage damage and exposure of the underlying subchondral bone) were comparatively rare.

The Gamma statistic was utilised to determine linear correlations between condylar pathology and POD lesion grades. Where significant this indicated that as the grade of POD increased, the grade of other pathology also increased in a linear fashion. It was found that a significant linear relationship existed between POD grade and grades of cartilage ulceration and wear lines, therefore supporting our hypothesis that increasing grades of POD are associated with progression of generalised joint disease within the MCP/MTP joint. However, we failed to show a relationship between POD and marginal remodelling. Only small numbers of condyles (5%) were recorded as being affected with marginal remodelling. It is likely that the true prevalence of marginal remodelling of the joint in general was underestimated as the margins of the condyle of MC/MTIII are less commonly affected than other sites within the joint (e.g. base of the proximal sesamoid bones). Failure to recognise marginal remodelling at post mortem may therefore explain the lack of significant correlation with POD. A further larger sample is now being examined, which includes scoring of marginal remodelling at other relevant sites within the joint.

In this study, the post mortem finding of "dorsal impact injury" encompassed both pathology of the dorsal synovial pad and associated damage to the underlying MC/MTIII. Both chronic proliferative synovitis and traumatic injury to the dorsodistal MC/MTIII are thought to occur in racehorses as a result of repeated overextension and cyclic impaction of the MCP/MTP joint (Pool 1996; Richardson 2003). The finding of a linear association between POD and dorsal impact injuries provides further evidence that POD occurs in association with cyclical repetitive overextension of the MCP/MTP joint as has previously been suggested by Pool (1996) and Norrdin et al. (1998).

The relationship between POD and linear fissures was found to be complex. Linear fissures are creases in the articular cartilage, affecting to a greater or lesser extent the underlying

subchondral bone and occur in the palmar/plantar aspect of the grooves on either side of the sagittal ridge of MC/MTIII (Riggs et al.1999). In this study there was a decreased severity of POD in animals with grade 1 linear fissures in comparison to horses with no evidence of linear fissures or grade 2 fissures. The reasons for this are unclear at present but may be associated with the biological response of subchondral bone to training. This requires further analysis with a larger sample of horses and further investigation of sections from the condyles of affected individuals using other laboratory methods such as scanning electron microscopy and gene expression techniques.

5.5 Conclusion

In conclusion, we have shown that POD in the racing Thoroughbred is common. The condition occurs in equal frequency between front and hind legs, and there is no left/right pre-disposition despite horses in this population training and racing predominantly in one direction. There is an association between severity of POD and other pathologies associated with generalised osteoarthritis. Further study is required to understand the basic pathogenesis of this common disorder and identify risk factors for its occurrence.

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Histological Appearance and Comparison of the OOCHAS and Mankin Scoring Systems in Grading of Cartilage Histopathology in Palmar Osteochondral Disease

6.1 Introduction

Palmar osteochondral disease (POD) is a condition affecting the palmar and plantar distal condyles of third metacarpal and metatarsal bones of horses and is found predominantly in racing breeds (Barr *et al.* 2009; Riggs 2006). In Chapter 5 (Palmar Osteochondral Disease as a Naturally Occurring Manifestation of SCB Driven Osteoarthritis in the Horse) and in the previous literature (Barr *et al.* 2009; Hornof *et al.* 1981; Pool 1996; Riggs 2006) the gross appearance of these lesions at post mortem examination have been described.

Given the considerable clinical importance attached to the adaptation of the subchondral bone of horses in response to exercise particularly in predilection sites such as the palmar/plantar condyle of the distal metacarpus/metatarsus, research interest in this area has increased over the past decade (Kawcak *et al.* 2000; Norrdin *et al.* 1998; Riggs and Boyde 1999). The calcified cartilage and subchondral bone of the distal metacarpal condyle of the horse has been investigated using various methods such as scanning electron microscopy (Doube *et al.* 2007; Norrdin and Stover 2006), confocal scanning light microscopy (Doube *et al.* 2007), micro-computed tomography (Rubio-Martinez *et al.* 2008), histology using basic fuschin staining of bone blocks (Muir *et al.* 2008), MRI (Martinelli *et al.* 1997; Sherlock *et al.* 2009)and computed tomography (Young *et al.* 2007).

The histological appearance of normal and osteoarthritic articular cartilage of the distal metacarpus of horses has been previously described (Smith *et al.* 2006), however this study made no reference to the underlying subchondral bone. A previous study (Hornof *et al.* 1981) has looked at the histological appearance of the lesions which we refer to as POD, however these authors described the lesions as a manifestation of osteochondritis dissecans. More recently it has become generally

believed that these lesions in fact arise as a result of trauma and overload of the palmar/plantar condyle (Barr *et al.* 2009; Pool 1996; Riggs 2006). A study describing the histological appearance of POD lesions as manifestations of trauma using a quantifiable system for assessing articular cartilage and subchondral bone has not been performed to our knowledge.

Various grading systems have been developed and utilised for assessment of osteoarthritis (OA). One of the most widely used of these is that developed by Mankin et al. (1971). Mankin's system uses a 14 point score based on a composite of cellular changes, histochemical presence of Safranin O matrix staining and architectural changes (e.g. erosion, vessel penetration through the tidemark). This system, or an adapted version of this system, has been used widely by most investigators. However, as the system was developed on samples with very advanced OA, it has been found that the Mankin grading system is not linear for mild or early phase disease (Ostergaard *et al.* 1999; Ostergaard *et al.* 1997; van der Sluijs *et al.* 1992). In order to standardise the assessment of OA, an Osteoarthritis Research Society International (OARSI) Working Group developed the OARSI Osteoarthritis Cartilage Histopathology Assessment System (OOCHAS) (Pritzker *et al.* 2006). The OOCHAS assigns an overall score that combines an OA grade (0-6 points) and OA stage (0-4 points), representing a combined assessment of OA severity and extent (0-24 points). The OOCHAS has been found to have higher reliability than the Mankin scoring system, although both have excellent intra- and inter-observer reproducibility and variability and a good positive correlation between the scores (Custers *et al.* 2007).

In the human, synovial biopsy is widely regarded to have a small role in the eventual diagnosis of difficult undiagnosed monoarticular arthritis, in those cases where synovial fluid analysis and other examinations have been non-diagnostic (Bywaters 1985). The value of synovial biopsy in differential diagnosis of various rheumatic diseases has been reported to be limited since histopathologic findings are often non-specific (Sherman 1951) however other authors (Goldenberg and Cohen

1978) have reported that although histopathologic changes in the common rheumatic diseases (rheumatoid arthritis, gout, pseudogout, systemic lupus erythematosus, infectious arthritis and osteoarthritis) are not specific, they are of diagnostic utility. Synovial membrane biopsy is not routinely used as a diagnostic procedure in the equine and there is therefore a paucity of published data on the appearance of this tissue in disease.

The aim of the investigations described in this chapter was to describe the histological appearance of cartilage, subchondral bone and synovial membrane collected from a population of Thoroughbreds which had been in flat race training, and a number of which were known to be affected by POD based on *post mortem* examination. Two commonly used scoring systems for cartilage pathology (Mankin and OOCHAS) were applied to the samples in an attempt to compare reliability of the two scoring systems and to ascertain which was most appropriate for use in analysis of cartilage pathology in samples of palmar and plantar condyles from horses affected by POD.

It was hypothesised that POD would have a characteristic and well-defined histological appearance and that the OOCHAS scoring system would be more reliable in analysis of cartilage pathology in samples of condyles affected by POD.

6.2 Materials and Methods

Material was collected at post-mortem examination from a population of Thoroughbred racehorses that were in active race training or had been retired from active race training at the Hong Kong Jockey Club.

The distal articular surface of MC/MTIII was examined by gross observation at *post mortem* examination performed immediately after death as described in full in Chapter 5. All condyles were photographed immediately after disarticulation of the metacarpo-/metatarsophalangeal joint. A

scoring system was developed and each condyle was assigned a score for POD from 0-3 as outlined in Table 6.1. Sections of palmar/plantar condyle were obtained from the left medial distal metacarpal condyle and the left lateral metatarsal condyles. Material collection and gross *post-mortem* grading were performed by collaborators at the Hong Kong Jockey Club (Dr Christopher Riggs, Peter Curl and Suzanne Troester).

Score	Description
0	No evidence of POD
1	Discolouration (bruising) of subchondral bone only. No or minimal disruption to the overlying cartilage
2	Discolouration (bruising) with mild to moderate disruption of the articular cartilage
3	Established POD lesions. Discolouration and disruption/collapse of the articular surface

Table 6.1: Gross Post Mortem Scoring System for Palmar Osteochondral Disease (POD)

After gross examination, the condyle was cut with a band saw, then two sections, each 2mm thick, were cut with a saline cooled diamond saw. Sections of synovial membrane were obtained from the dorsal aspects of the left metacarpophalangeal and metatarsophalangeal joints. Both osteochondral and synovial membrane samples were fixed in formalin for 48 hours then stored in ethanol. Osteochondral samples were decalcified in EDTA, paraffin embedded, sectioned at 6μm and stained with Haematoxylin & Eosin (H&E), Safranin O and Prussian Blue stains. Synovial membrane samples were paraffin embedded, sectioned at 6μm and stained with H&E. Histopathology preparation was performed by Sean Williams, University of Liverpool.

Sections were examined under an Eclipse 80i (Nikon UK Limited, Kingston upon Thames, UK) light microscope at magnifications of x4 and x10. Images were obtained using a DS-U1 camera (Nikon UK Limited, Kingston upon Thames, UK). Articular cartilage histopathology was quantified using the Histologic/Histochemical Grading System as described by Mankin et al. (1971) (Figure 6.1) and the OOCHAS scoring system (Pritzker *et al.* 2006) (Figure 6.2). Synovial membrane samples were examined to determine degree of inflammation based on the criteria described by Goldenberg and Cohen (1978). Cartilage, subchondral bone and synovial membrane histological findings were also

recorded in a descriptive fashion. Assistance in interpretation of histopathological findings was provided by Dr Udo Hetzel, University of Liverpool.

I Structure	
A Normal	0
B Surface Irregularities	1
C Pannus and surface irregularities	2
D Clefts to transitional zone	3
E Clefts to radial zone	4
F Clefts to calcified zone	5
G Complete disorganisation	6
II Cells	
A Normal	0
B Diffuse hypercellularity	1
C Cloning	2
D Hypocellularity	3
III Safranin O staining	
A Normal	0
B Slight reduction	1
C Moderate reduction	2
D Severe reduction	3
E No dye noted	4
IV Tidemark integrity	
A Intact	0
B Crossed by blood vessels	1
Total score	
Minimal	**0**
Maximal	**14**

Figure 6.1: Mankin Scoring System (Mankin et al. 1971)

I – Grade = depth progression into cartilage

Grade (key feature)	Subgrade (optional)	Associated criteria (tissue reaction)
Grade 0: surface intact, cartilage intact	No subgrade	Intact, uninvolved cartilage
Grade 1: surface intact	1.0 cells intact 1.5 cell death	Matrix: superficial zone intact edema and/or fibrillation Cells: proliferation (clusters), hypertrophy Reaction must be more than superficial fibrillation only
Grade 2: surface discontinuity	2.0 Fibrillation through superficial zone 2.5 Surface abrasion with matrix loss within superficial zone	As above + Discontinuity at superficial zone +/- Cationic stain matrix depletion (Safranin O or Toluidine Blue) upper 1/3 of cartilage (mid zone) +/- Disorientation of chondron columns
Grade 3: vertical fissures	3.0 Simple fissures 3.5 Branched/complex fissures	As above + Cationic stain depletion (Safranin O or Toluidine Blue) into lower 2/3 of cartilage (deep zone) +/- New collagen formation (polarized light microscopy, Picro Sirius Red stain)
Grade 4: erosion	4.0 Superficial zone delamination 4.5 Mid zone excavation cartilage matrix	Cartilage matrix loss, cyst formation within cartilage matrix
Grade 5: denudation	5.0 Bone surface intact 5.5 Reparative tissue surface present	Surface is sclerotic bone or reparative tissue including fibrocartilage
Grade 6: deformation	6.0 Joint margin osteophytes 6.5 Joint margin and central osteophytes	Bone remodelling. Deformation of articular surface contour (more than osteophyte formation only) Includes: microfracture and repair

II – Stage = extent of joint/sample involvement

Stage	% Involvement (surface, area, volume)
Stage 0	No OA activity seen
Stage 1	<10%
Stage 2	10-25%
Stage 3	25-50%
Stage 4	>50%

III Score – semi-quantitative method

Grade	Stage			
	S1	S2	S3	S4
G1	1	2	3	4
G2	2	4	6	8
G3	3	6	9	12
G4	4	8	12	16
G5	5	10	15	20
G6	6	12	18	24

Figure6. 2: OOCHAS System (Pritzker et al. 2006)

As outlined in Figure 6.3, based on a combination of histopathological examination and gross examination at *post-mortem*, Osteochondral samples were given a final binary POD grade (0/1) indicating that the sample was either unaffected (0) or affected (1) by POD based on whether there was evidence of POD on gross examination on *post mortem* examination and/or histopathological evidence of POD.

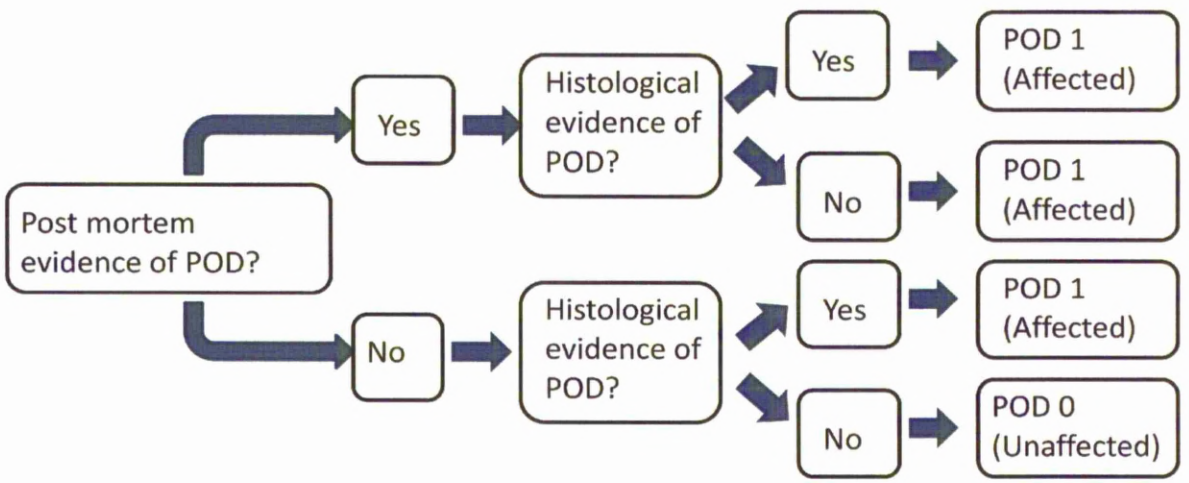

Figure 6.3: Flow diagram showing classification of samples into final grading for affected (1) or unaffected (0) for POD based on post mortem and histopathological examination.

The histological appearance of POD is described in full in the results section and in Figures 6.9-6.16, but briefly osteochondral samples were classified as having histological evidence of POD where there was fragmentation of the subchondral bone evident in the H&E stained section +/- evidence of increased Safranin O staining in the subchondral bone indicating cartilaginous elements (most likely fibrocartilage) within the subchondral bone plate. Loss of Safranin O staining from the hyaline

articular cartilage was seen in sections affected by POD, but as this demonstrated loss of proteoglycans from the articular cartilage, this was a non-specific indicator of osteoarthritis and not a histological indicator of POD per se.

6.3 Results

Twenty four condyles and twenty four synovial membrane samples were collected from 12 horses. All horses were Thoroughbred racehorses that had been in flat race training.

Articular cartilage pathology was quantified using the Mankin and OOCHAS scoring systems. Using the Mankin scoring system, mean score was 4.2 (range 0-12). Using the OOCHAS scoring system, mean score was 7.7 (range 0-24). Spread of data for the Mankin and OOCHAS scoring systems respectively is as shown in Figures 6.4 and 6.5. Using the Mankin scoring system, data was clustered towards the lower and upper ends of the scaling system, with relatively fewer samples having scores in the middle ranges of the system. Using the OOCHAS, the spread of data was slightly more even, although there was still some clustering at the lower and upper limits of the scoring sytem. Two samples had Grade 1 pathology using the Mankin scoring system, but Grade 0 on the OOCHAS scoring system as a result of slight reduction in Safranin O staining and superficial fibrillation of the cartilage surface which were sufficient to achieve 1 point on the Mankin scoring system, but not sufficient to attain Grade 1 using the OOCHAS scoring system.

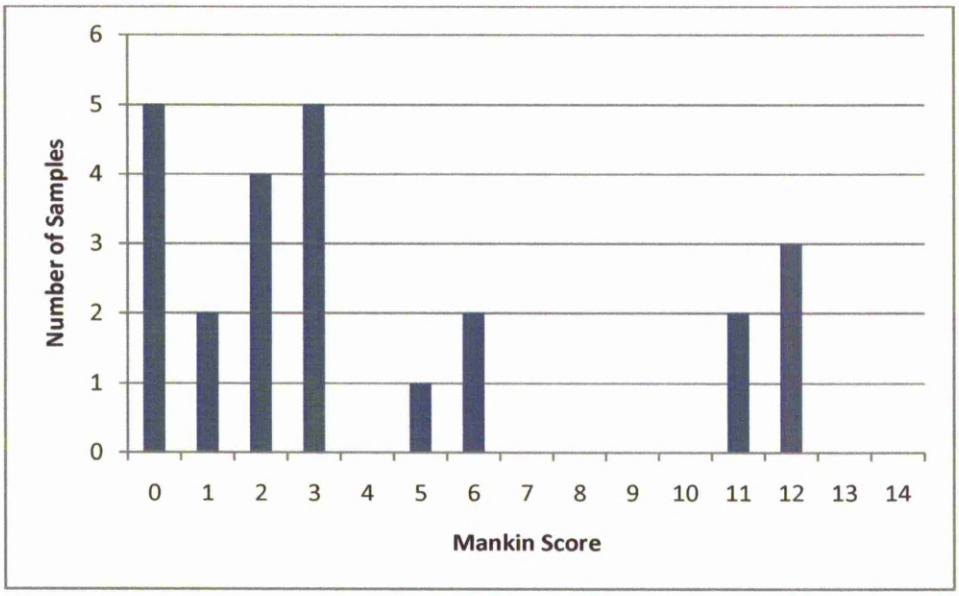

Figure 6.4: Spread of Data for Mankin Scoring System

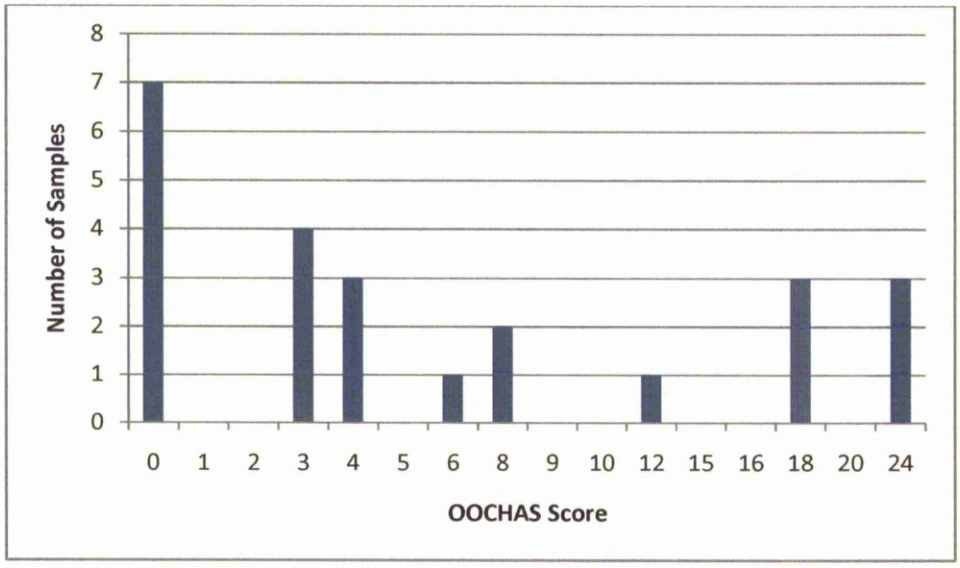

Figure 6.5: Spread of Data for OOCHAS Scoring System

Data for donor, limb, gross POD score, Mankin score, OOCHAS score and POD histology grade are as shown in Table 6.2.

Donor	Limb	Gross POD Grade (0-3)	Mankin Grade (0-14)	OOCHAS Grade (0-24)	Final POD Grade (0-1)
1	LF	0	2	3	0
2	LF	3	12	24	1
3	LF	0	3	3	0
4	LF	0	3	8	0
5	LF	0	1	0	0
6	LF	1	0	0	1
7	LF	0	1	0	0
8	LF	0	3	4	0
9	LF	0	2	3	0
10	LF	3	11	24	1
11	LF	0	2	3	0
12	LF	2	12	18	1
1	LH	0	0	0	0
2	LH	0	5	6	0
3	LH	0	6	18	1
4	LH	0	12	18	1
5	LH	0	2	4	0
6	LH	3	6	12	1
7	LH	0	0	0	0
8	LH	0	0	0	0
9	LH	2	3	4	1
10	LH	3	11	24	1
11	LH	0	3	8	0
12	LH	0	0	0	0

Table 6.2: Donor, Limb, Gross POD Grade, Mankin Grade, OOCHAS Grade and Final POD Grade for each sample

Correlation analysis using Pearson correlation revealed a significant correlation between the grade of POD pathology given to the sample on gross examination at post-mortem and both the Mankin scoring system ($P<0.001$, $r=0.7$) and the OOCHAS scoring system ($P<0.001$, $r=0.7$) (Figures 6.6 and 6.7).

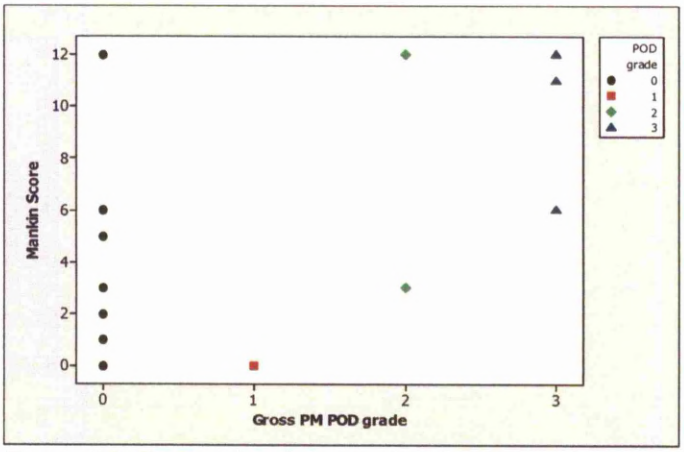

Figure 6.6: Scatterplot showing significant correlation between gross score for palmar osteochondral disease at post mortem examination and Mankin score on histopathological examination (P<0.001, r=0.7)

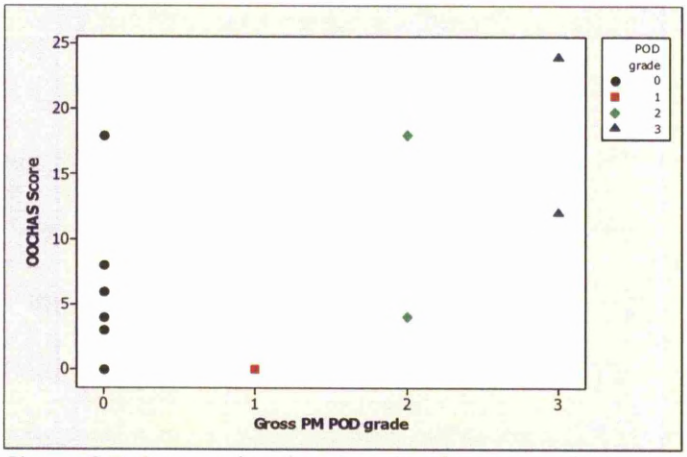

Figure 6.7: Scatterplot showing significant correlation between gross score for palmar osteochondral disease at post mortem examination and OOCHAS score on histopathological examination (P<0.001, r=0.7)

Correlation analysis using Pearson correlation between the Mankin scoring system and OOCHAS revealed a significant correlation between the two scoring systems (P<0.001, r=0.95) (Figure 6.8).

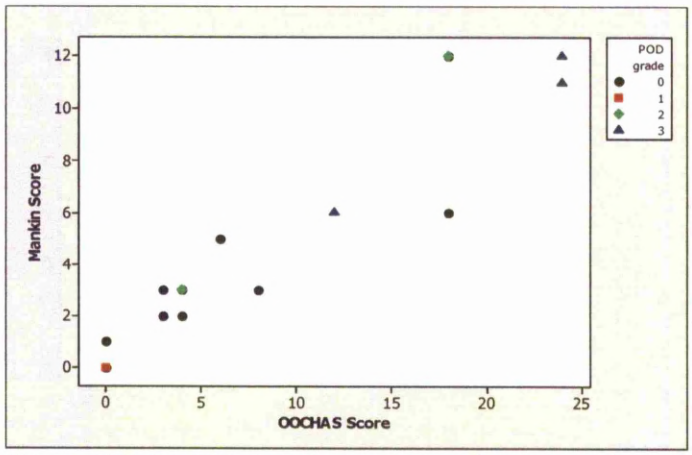

Figure 6.8: Scatterplot showing significant correlation between Mankin Score and OOCHAS on histological examination (P<0.001, r=0.95)

Palmar osteochondral disease was evident histologically predominantly based on the appearance of the subchondral bone. In the severely affected sections (POD grades 2 and 3 at gross *post mortem* examination) there was obvious fragmentation of the subchondral bone (Figures 6.9 and 6.10). Dependent on the severity of the POD lesion, the overlying articular cartilage was either intact (Figure 6.9) or had completely lost its architecture and had collapsed (Figure 6.10). There was loss of Safranin O staining in the superficial layer of articular cartilage in all sections with evidence of subchondral bone fragmentation, indicating proteoglycan loss from the articular cartilage matrix. This was more severe in sections with collapse of the articular surface (Figures 6.9 and 6.10).

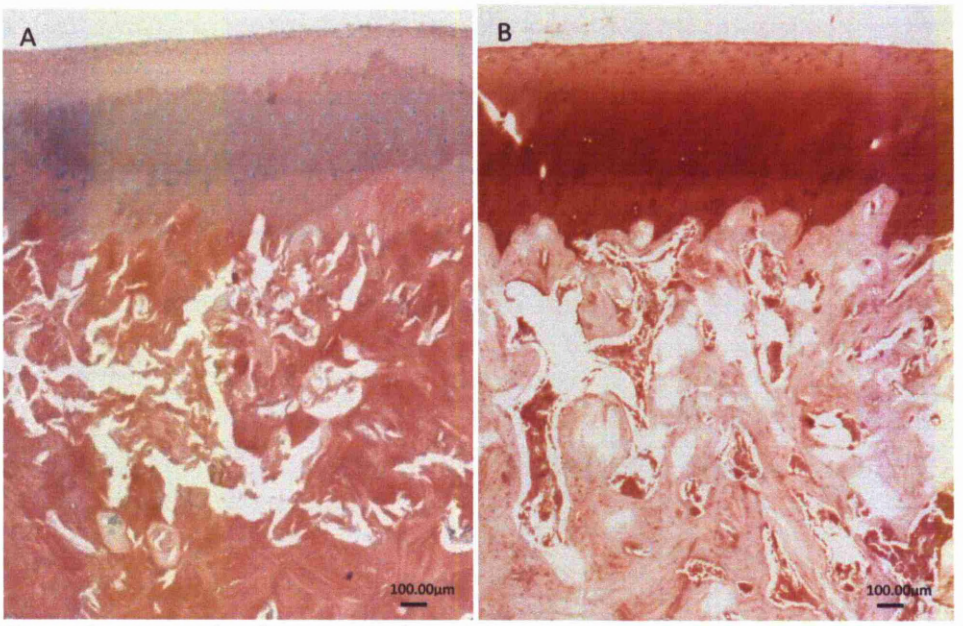

Figure 6.9: Example of a section of condyle with evidence of POD but intact articular cartilage. In the H&E section at x4 magnification (A) there is fragmentation of the subchondral bone plate with fibrocartilage tissue formation between the osseous fragments. In the Safranin O stained section at x4 magnification (B) there is a mild reduction in Safranin O staining in the superficial layer of cartilage and deep red Safranin O staining within the subchondral bone plate indicating cartilaginous elements within the subchondral bone plate.

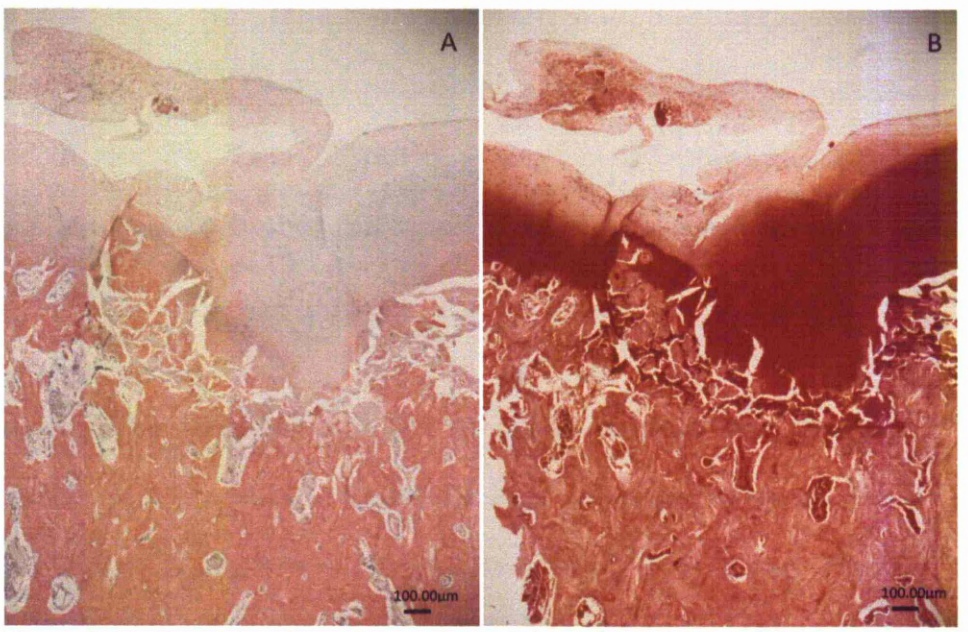

Figure 6.10: Example of a section of condyle with evidence of POD and complete collapse of the overlying articular cartilage. On the H&E section at x4 magnification (A) there is severe subchondral bone fragmentation and complete loss of architecture of the overlying articular cartilage. A normal tidemark is no longer identifiable. On the Safranin O section at x4 magnification (B) there is a marked reduction in staining in the superficial articular cartilage and deep red Safranin O staining in areas of the fragmented subchondral bone plate.

At higher magnification (x10) H&E sections it was possible to visualise areas of tidemark (transition zone between mineralised and non-mineralised cartilage) abnormalities. In some areas tidemark integrity had been lost and clefts ran across from the subchondral bone to the calcified articular cartilage and the deeper layers of the hyaline articular cartilage. There was evidence of debris within the marrow spaces. Duplication of the tidemark was also a common finding in sections affected by POD (Figure 6.11).

Figure 6.11: An example of an H&E section at x10 magnification showing loss of tidemark integrity with clefts running from the subchondral bone into the calcified cartilage and deep layers of articular cartilage. There is debris within the marrow spaces. Duplication of the tidemark is also evident on this section.

Also at higher magnification, it was possible to appreciate that there was an amorphous cellular infiltrate with an extracellular matrix between the osseous fragments. This tissue stained positively with Safranin O indicating cartilaginous elements within this tissue. This tissue was likely therefore to be consistent with granulation tissue and fibrocartilage formation, potentially as part of a fracture callus and an attempt at healing of the fractured subchondral bone plate (Figure 6.12).

Figure 6.12: An H&E section at x10 magnification showing evidence of a cellular infiltrate with an amorphous extracellular matrix between the fragments of subchondral bone plate and debris within the marrow spaces. This may be consistent with granulation tissue and fibrocartilage formation i.e. callus formation and potentially an attempt at fracture healing. Duplication of the tidemark is also shown on this section.

At higher magnification again (x40) it was possible to identify osteoclasts actively removing devitalised bone (Figure 13). It is suggested that the bone is necrotic in these areas due to the presence of empty osteocytic lacunae and because the bone matrix is more pale staining than in a normal section of subchondral bone (Figure 6.13).

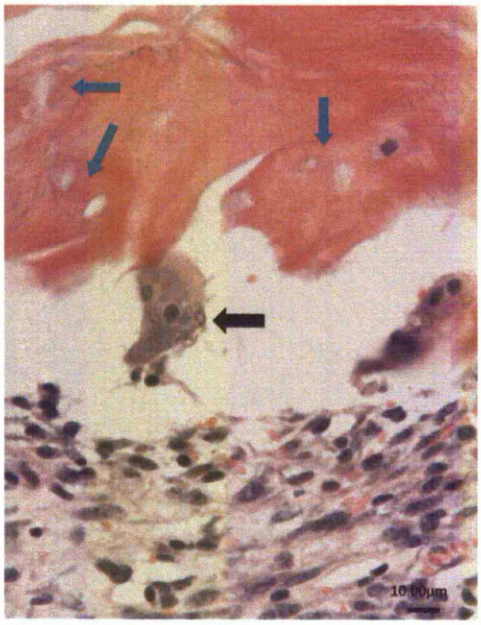

Figure 6.13: An H&E section at x40 magnification showing evidence of an osteoclast (black arrow) actively removing devitalised bone. Numerous empty lacunae are evident within the piece of bone (blue arrows) and the bone matrix is pale staining compared to normal.

Although the samples graded as 2 or 3 at *post mortem* examination all showed histological evidence of POD as described above, the one sample which was graded grossly as grade 1 did not show these changes in the subchondral bone (Figure 6. 14). On H&E staining of the grade 1 section, there was no evidence of fragmentation of the subchondral bone and no loss of Safranin O staining in the hyaline articular cartilage, indicating no evidence of loss of proteoglycans from the cartilage matrix. The grade 1 section showed evidence of positive Prussian blue staining, indicating haemosiderin in the section and therefore the presence of old haemorrhage in the tissue. None of the other sections examined showed evidence of positive Prussian blue staining.

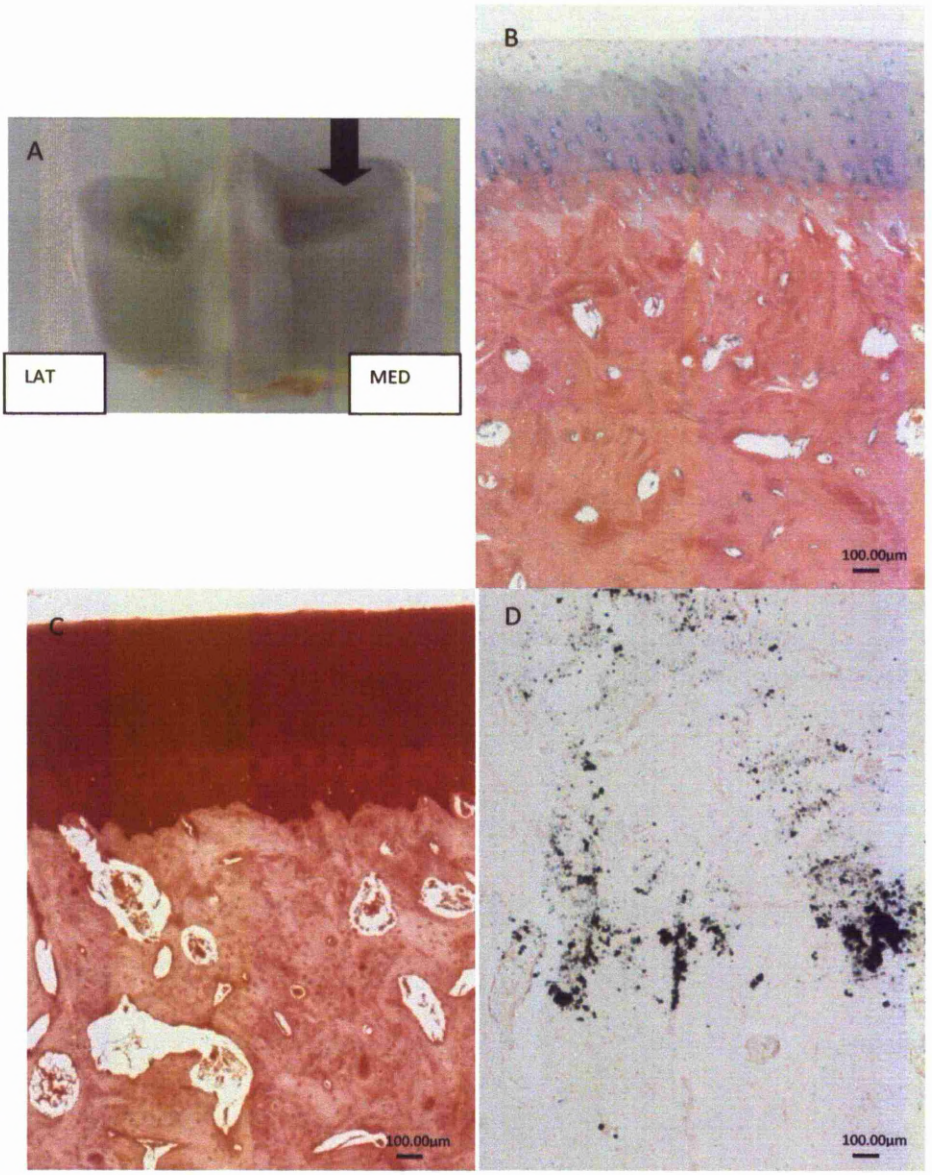

Figure 6.14: Gross post mortem picture of a distal MCIII condyle with Grade 1 POD on the medial condyle (arrow) (A) and corresponding H&E (B), Safranin O (C) and Prussian Blue sections (D). All sections at x4 magnification. Although bruising of the subchondral bone is evident on gross examination, there is no fragmentation of the subchondral bone or loss of proteoglycan staining. There is evidence of previous haemorrhage in the subchondral bone shown by positive Prussian Blue staining.

Of the 17 condyles graded as 0 for POD on gross examination, only 15 of these samples showed no evidence of POD on histology. Re-examination of the original gross pathology scoring sheets and of the photographs taken at *post-mortem* examination revealed that these condyles although not showing classical POD lesions, were also not grossly normal. These condyles had a discrete area of opaque, smooth, well-integrated cartilage at the site of POD lesions of unknown aetiology as

described previously (Barr *et al.* 2009). On histological examination of the H&E sections, there was evidence of complete loss of the tidemark, increased vascular channels within the subchondral bone and fibrocartilage formation within the subchondral bone as described above (Figure 6.15). The overlying articular cartilage appeared to be well-integrated with the fibrocartilage in the subchondral bone defect, but was not normal hyaline cartilage, having a disorganised cartilage architecture and evidence of a loss of Safranin O staining (Figure 6.15). It was considered that these histological findings may be consistent with a previous POD lesion that had undergone healing by fibrocartilage formation. Therefore these sections were classified as being affected for POD in the final binary POD grading system.

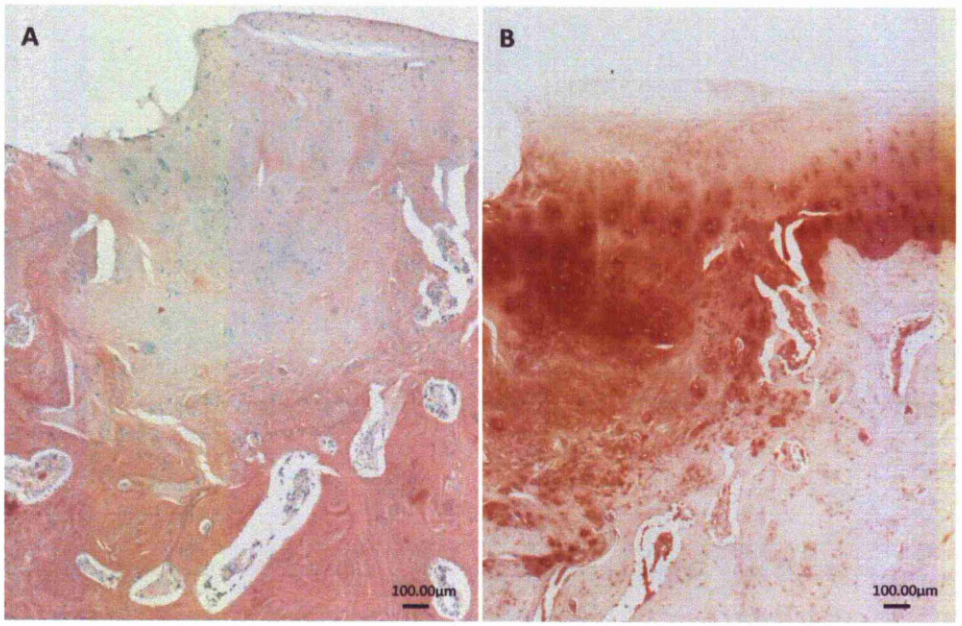

Figure 6.15: Example of a section of condyle classified as having a discrete area of opaque, smooth well-integrated cartilage at the site of POD lesions of unknown aetiology on gross post mortem examination on H&E section (A) and Safranin O staining (B) (both at x4 magnification). There is evidence of complete loss of the tidemark, increased vascular channels and fibrocartilage formation within the subchondral bone plate on the H&E section and a marked reduction in Safranin O staining indicating proteoglycan loss in the overlying articular cartilage. There is Safranin O staining of the tissue fibrocartilaginous tissue within the subchondral bone defect. These histological changes may be indicative of a previous POD lesion that has attempted healing by fibrocartilage formation within the fractured subchondral bone.

Mild cartilage pathology characterised by fibrillation of the superficial zone of articular cartilage with depletion of Safranin O staining was common on the palmar and plantar distal MC/MT III condyles in this sample of Thoroughbreds in training (Figure 6.16). Of all samples (n=24), including the samples classified as being unaffected for POD on final grading (n=15), only 4 had a Mankin and OOCHAS Grade 0 score.

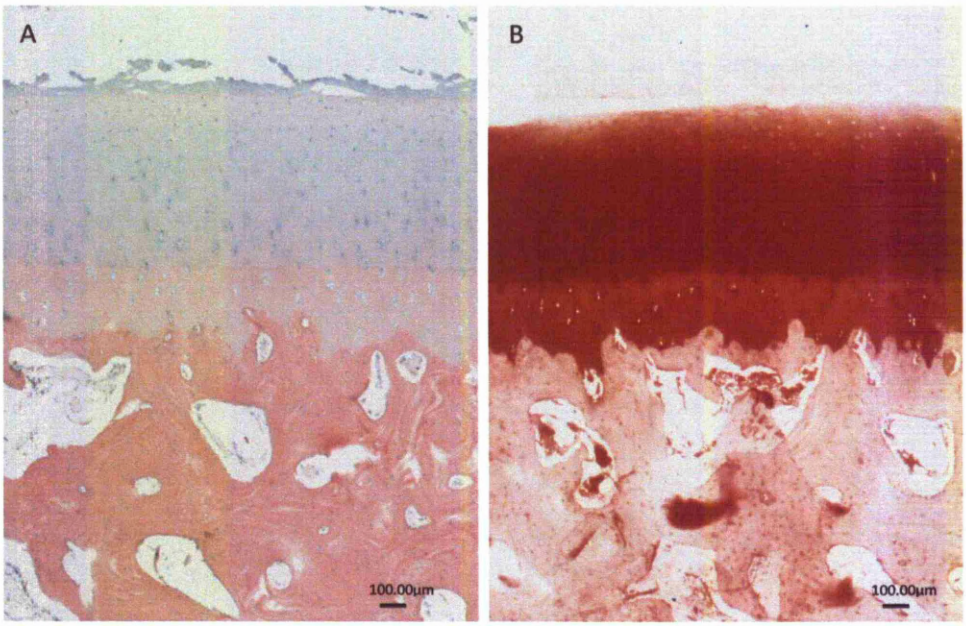

Figure 6.16: Example of a section of condyle without evidence of POD, but with fibrillation of the superficial zone of cartilage on H&E staining (A) and slight reduction of Safranin O staining (B). Both at x4 magnification.

The samples graded as 0 on the final POD score had a mean Mankin score of 1.8 (range 0-5) and a mean OOCHAS score of 2.8 (range 0-8). Samples classified as being affected for POD on final grading (n=9) had a mean Mankin score of 8.1 (range 0-12) and a mean OOCHAS score of 15.8 (range 0-24).

Twenty two/24 synovial membrane samples showed evidence of mild villous hyperplasia and mild perivascular lymphocytic synovitis, which were considered to be within normal limits for this population of horses (Figures 6.17 and 6.18). One synovial membrane section showed evidence of moderate villous hyperplasia (Figure 6.17) and one had evidence of a moderate-marked perivascular lymphocytic synovitis (Figure 6.18). No significant correlations between synovial membrane

histopathology and palmar osteochondral disease could be identified due to the small number of abnormal samples.

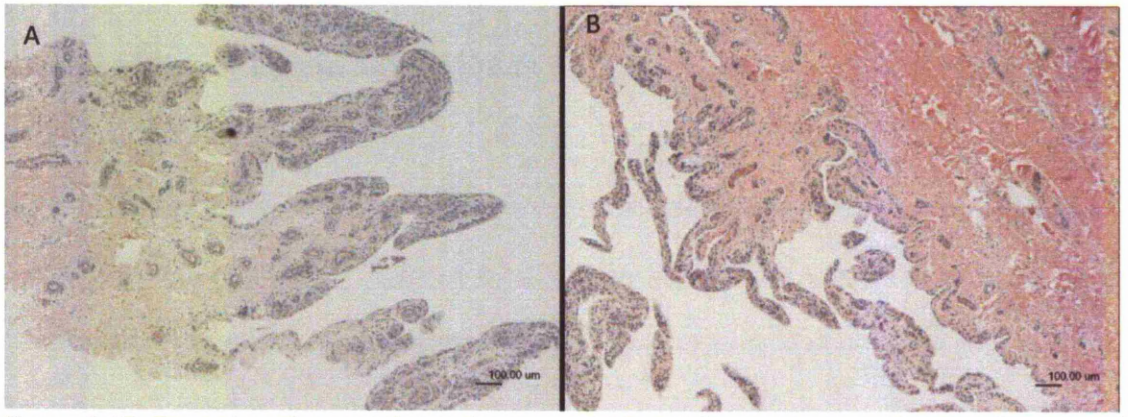

Figure 6.17: Examples of synovial membrane on H&E staining at x4 magnification showing (A) mild villous hyperplasia of the synovial membrane (B) moderate villous hyperplasia of the synovial membrane

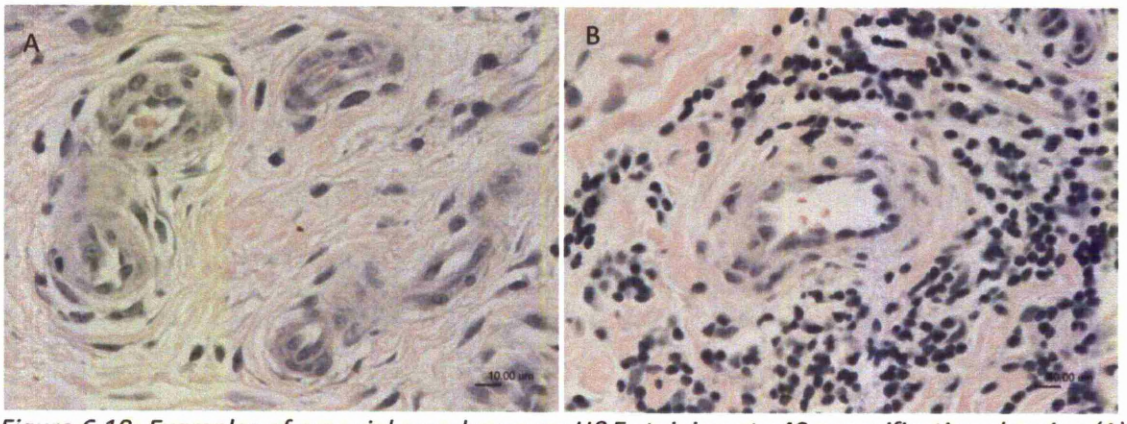

Figure 6.18: Examples of synovial membrane on H&E staining at x40 magnification showing (A) mild perivascular lymphocytic synovitis and (B) moderate perivascular lymphocytic synovitis

6.4 Discussion

It has been shown that osteochondral samples affected by more severe grades of POD on gross *post mortem* examination have a readily identifiable histopathological appearance with fracture and fragmentation of the subchondral bone plate. Our histological findings were very similar to those described by Hornof *et al.* (1981) with the added interesting finding of two samples in which the subchondral defect had appeared to heal with fibrocartilage, which in turn had attempted integration with the hyaline articular cartilage. This would suggest that these lesions are in fact able

to heal to some extent, albeit with a tissue which may be inferior to the original subchondral bone and hyaline articular cartilage. The appearance of the fractured subchondral bone plate with callus formation (granulation tissue and fibrocartilage) would also support a traumatic aetiology for POD as has been previously proposed by Pool (1996).

Histopathology of the palmar metacarpal condyle of the equine has been described in a recent paper by Drum et al. (2007). Drum et al. developed a grading system for severity of lesions on a scale of 0-4. Based on their description of the grading system, it would seem that their histology findings were similar to ours, with evidence of duplication of the tidemark and reparative fibrocartilage in the superficial cartilage layers. Drum et al. described pale staining of the subchondral bone matrix and fibrin within the subchondral bone layer, whereas I described similar findings as fibrocartilage formation within the subchondral bone. Differences may be due to personal preference in description of histopathological lesions and because in the paper by Drum et al. (2007) no severely affected samples were observed. I did observe sections of more severely affected palmar condyles and therefore was able to provide a more detailed description of these lesions.

The histopathological appearance of the lesions as shown in Figure 6.11 and described as loss of tidemark integrity with clefts running across the subchondral bone into the calcified cartilage and deep layers of articular cartilage, have also been described in the human osteoarthritis literature under a number of synonymous names including vascular invasion (Harrison *et al.* 1953), vascular channels (Clark 1990), microcracks (Mori *et al.* 1993; Sokoloff 1993) and subchondral bone resorption pits (Chambers *et al.* 1984). Subchondral bone resorption pits have previously been implicated in cartilage nutrition and cross-talk between subchondral bone and cartilage (Lajeunesse and Reboul 2003) and recently have been shown to express MMPs and degrade proteoglycan from the articular cartilage around the tip of the invasion (Shibakawa *et al.* 2005). The histological finding

of subchondral bone pits in sections of palmar condyle affected by POD when considered with the findings of these previous studies, provides more support for the role of subchondral bone damage resulting in cartilage matrix proteolysis in horses affected by POD.

It was suggested that some areas of osteonecrosis were evident within subchondral bone affected by POD as identified by empty osteocytic lacunae. Empty lacunae have been used as an indicator of osteonecrosis by previous investigators (Humphreys *et al.* 1989) however, some caution must be exercised in interpreting empty osteocytic lacunae as always being representative of osteonecrosis. This criterion of bone death has previously been criticised on the basis that artefactual loss of cells may occur during tissue processing and that nuclei may remain within lacunae long after cell death has occurred (Kenzora *et al.* 1978). However, in this case it is thought reasonable to suggest that osteonecrosis is histologically evident in these samples given that there is also evidence of osteoclasts resorbing the devitalised bone and because samples were handled identically and extensive loss of osteocytes from their lacunae was not seen in subchondral bone not affected by POD.

Unfortunately only one sample categorised grossly as Grade 1 was available for histopathological analysis in this study. It was interesting that in this case the subchondral bone bruising evident on gross examination was shown histologically as positive staining for Prussian Blue, indicating haemosiderin and therefore previous haemorrhage within the tissue. However, due to the fact that only one sample of this *post mortem* grade was available, no real conclusions can be drawn from this. Histopathological examination of more Grade 1 samples will be required to assess the importance of this finding, and this is ongoing as part of a larger study.

The finding of some degree of cartilage pathology on the palmar/plantar condyle of the distal metacarpus/-tarsus evident in the samples unaffected by POD is consistent with the findings of

Smith et al. (2006) who reported that degenerative changes on histology were most marked in the palmar region of condyles affected by osteoarthritis and that mild degeneration was evident in the palmar regions of control (non OA) condyles.

Cartilage pathology was quantified using both the Mankin and OOCHAS scoring systems. For grading POD, the main advantage of the OOCHAS over the Mankin system is that it allows for pathology within the subchondral bone plate, including a grading classification for microfracture and fibrocartilaginous repair. As well as providing a grade of osteoarthritis cartilage histopathology, the OOCHAS also allows for staging of the disease, quantified as the horizontal extent of cartilage involvement. Unfortunately, within the confines of this study, the samples available and the focal nature of POD, this staging was performed on the section of palmar/plantar of one condyle only, and not across the joint surface as described in the development of the system (Pritzker *et al.* 2006). Within this study however, this did not alter the results as the anatomical position and size of the section of condyle examined was the same in every case. Regardless of which scoring system for cartilage histopathology was utilised, it was found that both gave identical and significant correlations between histology score and grade of POD on gross examination of the sample at *post mortem* examination. This would suggest that both scoring systems are appropriate for use in quantative analysis of sections of equine palmar/plantar metacarpal/metatarsal condyles. This may not be surprising as the majority of the samples with POD examined here were severely affected. The Mankin grading system has been found to be useful for samples with advanced OA, but more limited when utilised on more mildly affected samples (Ostergaard *et al.* 1999; Ostergaard *et al.* 1997; van der Sluijs *et al.* 1992). As discussed above, the study is ongoing and larger numbers of early/mildly affected (Grade 1 on *post mortem* examination) samples are required and thus future comparison of the grading systems on these more mildly affected samples will potentially be enlightening.

Histology of the synovial membrane was largely unremarkable in this group of horses and due to the small number of samples which were found to have histological changes in the synovial membrane, no significant correlations could be found between synovial membrane pathology and the presence or severity of POD. This may be because of the small numbers of samples involved in this initial study, or because the synovial membrane sample was collected from the dorsal aspect of the joint i.e. distant to the palmar/plantar aspect of the joint which is the site of POD lesions. If this is not merely an effect of insufficient sample numbers, it would appear that diffuse synovitis of the metacarpo-/metatarsophalangeal joint is not a feature of POD. A larger study is ongoing and should further clarify this finding.

6.5 Conclusion

In conclusion, the histological appearance of osteochondral sections from the palmar and plantar distal metacarpal and metatarsal condyles affected by POD has been described. Both the Mankin and OOCHAS scoring systems were found to be useful in scoring of pathology of the sections examined here. There is some evidence that the lesions are capable of healing by fibrocatilaginous tissue formation. Synovitis did not appear to be a feature of POD. A larger ongoing study should clarify these findings and also further characterise the less severe, and presumably earlier, POD lesions characterised by bruising of the subchondral bone plate without fragmentation and fracture formation.

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Association Between Markers of Osteoarthritis in Subchondral Bone, Cartilage and Synovial Membrane in Horses with Palmar Osteochondral Disease

7.1 Introduction

As described in Chapter 5 (Palmar Osteochondral Disease as a Naturally Occurring Manifestation of Subchondral Bone Driven Osteoarthritis in the Horse), palmar osteochondral disease (POD) is a condition affecting the palmar and plantar distal condyles of the third metacarpal and metatarsal bones of horses affecting predominantly racing breeds (Barr *et al.* 2009; Riggs 2006). The condition is characterised by small, ovoid defects of approximately 2-4mm diameter centred 5-8mm proximal to the transverse ridge and 3-15mm from the sagittal ridge on either the medial or lateral condylar surfaces (Pool 1996). Early in the course of the disease, only the subchondral bone is affected, with bluish discolouration visible through intact cartilage. More severe changes involve varying degrees of physical disruption of the subchondral bone with associated pathology of the overlying cartilage. Ultimately there may be complete collapse of the subchondral bone.

Proteolytic damage to the collagen fibrillar network of the cartilage extracellular matrix is a key and perhaps irreversible stage in the pathogenesis of osteoarthritis (Eyre 2004). Metalloproteinases are considered to play a principal role in the cleavage of matrix macromolecules, including type II collagen and the cartilage proteoglycan aggrecan. The collagenases (MMPs 1, 8, 13 and 14) are capable of cleaving type II collagen, resulting in denaturation of the α chains, thus rendering them susceptible to secondary cleavage by collagenases and other MMPs such as MMP3 (a stromelysin) and MMPs 2 and 9 (gelatinases) (Poole *et al.* 2007). Aggrecan is cleaved by various MMPs and by the aggrecanases ADAMTS4 and ADAMTS5.

The aim of the investigations described in this chapter was to further investigate the role of subchondral bone (SCB) in osteoarthritis (OA), this time in an *ex vivo* model using samples obtained from condyles affected and unaffected with POD. Quantitative expression of various genes involved

in matrix synthesis and degradation were determined in an attempt to further our understanding as to the underlying pathologies involved in osteochondral disease. Palmar osteochondral disease provides a unique opportunity to investigate this process as SCB injury appears to be the initiating factor of the pathology, occurring before cartilage degradation. Furthermore, POD is a commonly diagnosed condition in racing breeds occurring in a repeatable and well defined site which facilitates sample collection and allows for excellent characterisation of the samples.

It was hypothesised that 1) there would be increases in gene expression of matrix proteinases in SCB, hyaline articular cartilage (HAC) and synovial membrane affected by POD; 2) there would be alterations in expression of genes involved in matrix synthesis in SCB and HAC and 3) there would be correlations between expression of genes in the various articular tissues (SCB, HAC and synovial membrane).

7.2 Materials and Methods

7.2.1 Tissue Collection

Material was collected at *post-mortem* examination from a population of Thoroughbred racehorses that were in active race training or had been retired from active race training. Sample collection was in collaboration with Dr Chris Riggs, Suzanne Troester and Peter Curl of the Hong Kong Jockey Club as previously described. Condyles were scored grossly for grade of POD as described in full in Chapter 5 and as outlined in Table 7.1. Racing and veterinary treatment records for each horse were available from an existing database.

Score	Description
0	No evidence of POD
1	Discolouration (bruising) of subchondral bone only. No or minimal disruption to the overlying cartilage
2	Discolouration (bruising) with mild to moderate disruption of the articular cartilage
3	Established POD lesions. Discolouration and disruption/collapse of the articular surface

Table 7.1: Gross Post Mortem Scoring System for Palmar Osteochondral Disease (POD)

Sections of palmar/plantar condyle were obtained from the left medial distal metacarpal condyle and the left lateral metatarsal condyles as shown in Figure 7.1. The condyle was cut with a band saw, then two sections, each 2mm thick, were cut with a saline cooled diamond saw. Sections of synovial membrane were obtained from the dorsal aspects of the left metacarpophalangeal and metatarsophalangeal joints. Samples were stored in RNA later (Ambion, Applied Biosystems, Warrington, UK) at 4°C for 24 hours, and then stored at -80°C until further analysis.

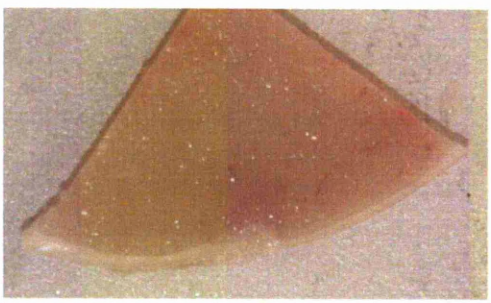

Figure 7.1: Example of a 2mm thick section of palmar condyle as obtained at sample collection

7.2.2. RNA Extraction and Reverse Transcription

Unless stated otherwise, all reagents were supplied by Sigma-Aldrich (Dorset, UK). Cartilage and SCB were dissected from the condyle sections. Cartilage was dissected sharply with a scalpel and a section of subchondral bone from the centre of the sample at the anatomical site of POD measuring approximately 15mm x 15mm was dissected using a chisel. Cartilage, SCB and synovial membrane were subsequently frozen in liquid nitrogen, dismembranated using a Mikro-Dismembrator U (Braun Biotech, Melsungen, Germany) and stored in Tri-Reagent (Ambion, Applied Biosystems, Warrington,

UK). RNA extraction was performed using a standard chloroform and ethanol extraction followed by the RNeasy (Qiagen, Crawley, UK) column technique, incorporating a DNase treatment stage. RNA was quantified using a Nanodrop (Thermo Scientific, Wilmington, USA) and 1μg of RNA or the maximum volume for the reaction (12.4μL) where RNA concentration was < 80.6ng/μL, was used as the template for the reverse transcriptase reaction. cDNA strands were generated from the RNA in a 25μL volume reaction using Random Primer (Promega, Southampton, UK), RT buffer, 10mM of dNTPs, M-MLV RT enzyme and RNase inhibitor.

7.2.3 Quantitative Real-Time Polymerase Chain Reaction

Equine specific primers were prepared as described in Chapter 2. Briefly, RNA and DNA sequences were obtained from the NCBI database, or where published equine sequence was unavailable, a multiple species alignment was performed. Primers were designed to be exon spanning where possible. Primers were designed using Primer Express software (Applied Biosystems) or Primer 3 software (Rozen and Skaletsky 2000). Primer efficiencies were validated using a standard curve derived from equine cDNA using a 10 fold dilution series. A dissociation curve was included in validation to ensure primers were specific and amplified only one product. Primer efficiency is optimal at a slope of -3.32. The primers used for the target and housekeeping genes and their efficiencies are as shown in Table 7.2.

Gene	Primer Sequence	Accession Code	Efficiency
GAPDH	F: GCATCGTGGAGGGACTCA R: GCCACATCTTCCCAGAGG	AF157626	-3.32
ADAMTS- 4	F: CAGCCTGGCTCCTTCAAAAA R: CCGCAGGAATAGTGACCACAT	NM_001111299	-3.23
ADAMTS- 5	F: ACCGATCCTGCAGTGTCACA R: CTGCTCATGGCGAAAAGATTT	EU025851	-3.13
MMP -1	F: GGTGAAGGAAGGTCAAGTTCTGAT R: AGTCTTCTACTTTGGAAAAGAGCTTCTCT	NM_001081847	-3.36
MMP- 3	F: TCTTGCCGGTCAGCTTCATATAT R: CCTATGGAAGGTGACTCCATGTG	NM_001082495	-3.63
MMP -13	F: CTGGAGCTGGGCACCTACTG R: ATTTGCCTGAGTCATTATGAACAAGAT	NM_001081804	-3.51
TIMP- 3	F: CTGCAACTTCGTGGAGAGGT R: ACTCGTTCTTGGAGGTCACG	NM_001081870	-3.54
Interleukin 1β	F: TGAAGGGCAGCTTCCAAGAC R: GGGAGAATTGAAGCTGGATGC	D42165	-3.34
Collagen I α2	F: GCACATGCCGTGACTTGAGA R: CATCCATAGTGCATCCTTGATTAGG	XM_001492939.1	-3.31
Collagen II α1	F: TCAAGTCCCTCAACAACCAGATC R: GTCAATCCAGTAGTCTCCGCTCTT	NM_001081764	-3.21
Collagen X α1	F: CAACATCAAGACCCGGTTCTTC R: CACCTTGTTCTCCTCTCACTGATACA	XM_001504101	-3.22
Biglycan	F: TCACCTTCCAGCCCCTAGAGT R: AGAAGCAGCCCCTCCTCAA	NM_001081839	-3.71
Aggrecan	F: GAGGAGCAGGAGTTTGTCAACA R: CCCTTCGATGGTCCTGTCAT	XM_001499504	-3.81

Table 7.2: Primer sequences used for quantitative real-time PCR
F= Forward primer, R= Reverse primer

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 25μL volume using SYBR Green PCR master mix (Applied Biosystems) and 300nM primer concentration and processed by 7300 Real Time PCR system (Applied Biosystems). Conditions for amplification were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C for primer annealing and elongation. This was followed by a dissociation stage for 15 seconds at 95°C, 30 seconds at 60°C and 15 seconds at 95°C to ensure the presence of a single amplicon. Data was analysed using SDS software (Applied Biosystems). PCR products were measured and normalised against GAPDH as a housekeeping gene. Gene expression of *MMP1, MMP3, MMP13, ADAMTS4, ADAMTS5, TIMP3* and *IL1B* were quantified in cartilage, SCB and synovial membrane. In addition, gene expression of *COL1A2, COL2A1, COL10A1*, biglycan (*BGN*) and aggrecan (*ACAN*) were also measured in cartilage and SCB.

Primer validation, RNA extraction, reverse transcription and qRT-PCR of the initial 24 samples were performed by the first author. Subsequent RNA extraction, reverse transcription and qRT-PCR of further samples were performed in collaboration with Colette Redmond and Dr Benjamin McDermott, both of the University of Liverpool.

7.2.4 Statistical Analysis

Samples were grouped into grade of POD (0-3) based on grading at *post mortem* examination. After plotting for normality and logging data where required using Minitab v.15 (Minitab Inc., Pennsylvania, USA), mixed effects linear regression using SPlus v.6.1 (TIBCO Software Inc., California, USA) was used to test for significant differences between groups while allowing for the clustering of samples within horse. To allow for testing of multiple genes on the same sample, the P-value was adjusted using Sidak's formula (Sidak 1967):

$$\alpha_{PT} = 1 - (1-\alpha_{PF})^{1/c}$$

where α is the probability of making a Type I error, α_{PT} is the α per test, α_{PF} is the α per family of tests and c is the number of comparisons, in this case genes tested. Therefore if we assume that the required α_{PF} is 0.05, then:

$$\alpha_{PT} = 1 - (1-0.05)^{1/c}$$

Correlation analysis using Spearman's rank correlation co-efficient was performed to identify relationships between expression of each gene in the SCB, cartilage and synovial membrane using PASW Statistics 17 (Chicago, Illinois, USA). Statistical significance for correlation analysis was set at $P<0.05$.

Gene expression data are presented as a box and whisker chart, with tops and bottoms of boxes demonstrating third and first quartiles respectively. The line within the box indicates the median value. Whiskers extend to the highest and lowest data values. Correlation of gene expression data between tissues is presented as scatter plots using a log scale.

7.3 Results

7.3.1 Sample Characterisation

Seventy condyles and 70 synovial membrane samples were collected from 35 horses. All horses were Thoroughbreds which had been in flat race training. Thirty four condyles (48.6%) had Grade 0 POD, 18 condyles (25.7%) had Grade 1 POD, 11 condyles (15.7%) had Grade 2 POD and 7 condyles (10%) had Grade 3 POD.

7.3.2 Alterations in Gene Expression in Palmar Osteochondral Disease

Seven genes were measured in synovial membrane and 12 genes were measured in cartilage and subchondral bone, giving a Sidak corrected significance level of $P \leq 0.007$ for synovial membrane samples and $P \leq 0.004$ for cartilage and subchondral bone samples.

Cartilage

In cartilage, as compared to samples unaffected by POD (Grade 0), there was a significant increase in expression of: *MMP13* in POD Grade 3 samples (P=0.0001) (Figure 7.2) and *COL1A2* in both POD Grade 2 (P=0.0001) and POD Grade 3 (P=0.0001) samples (Figure 7.3). There was a trend towards an increase in *MMP13* expression in POD Grade 2 samples (P=0.007) but this just failed to reach statistical significance.

Compared to POD Grade 0 samples, there was a significant decrease in expression of *ACAN* in POD Grade 1 (P=0.002), POD Grade 2 (P=0.002) and POD Grade 3 (P=0.0004) samples (Figure 7.4). There was a trend towards a decrease in *TIMP3* expression in Grade 1 POD samples (P=0.005).

Significance of differences in gene expression in cartilage of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD are shown in full in Table 7.3.

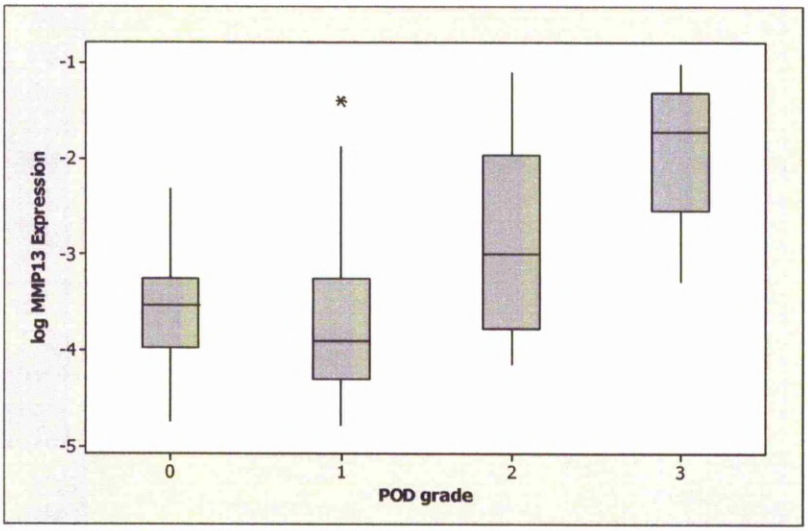

Figure 7.2: Box and whisker plot of MMP13 expression in articular cartilage affected by each grade of POD. Asterisks show outlying results. Expression of MMP13 was significantly increased in cartilage from Grade 3 POD samples (P=0.0001) and there was a trend towards an increase in MMP13 expression in Grade 2 POD samples (P=0.007).

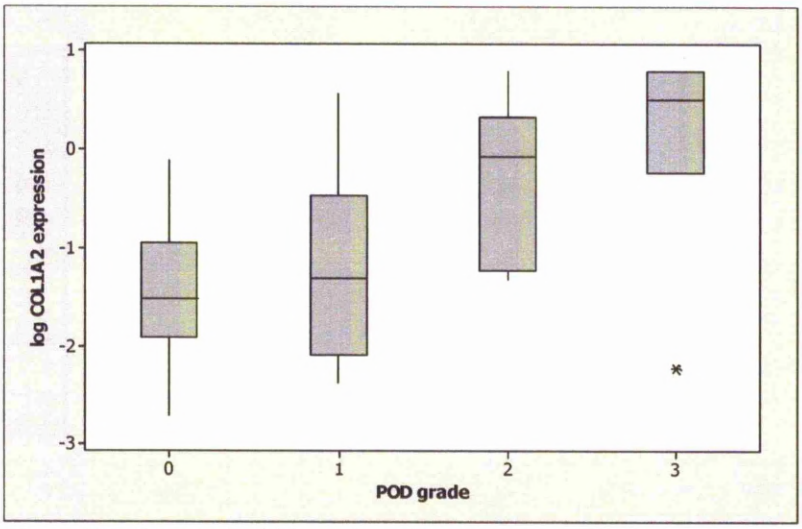

Figure 7.3: Box and whisker plot of COL1A2 expression in articular cartilage affected by each grade of POD. Asterisks mark outlying results. Expression of COL1A2 was significantly increased in cartilage from Grade 2 POD samples (P=0.0001) and Grade 3 POD samples (P=0.0001).

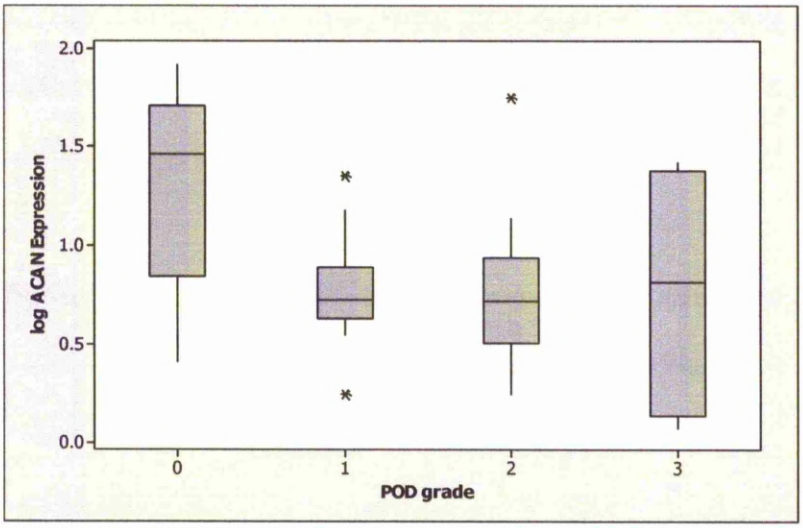

Figure 7.4: Box and whisker plot of ACAN expression in articular cartilage affected by each grade of POD. Asterisks show outlying results. Expression of ACAN was significantly decreased in cartilage from Grade 1 POD samples (P=0.002). Grade 2 POD samples (P=0.002) and Grade 3 POD samples (P=0.0004).

Cartilage			
Gene	POD Grade 1	POD Grade 2	POD Grade 3
ADAMTS4	0.06	0.2	0.3
ADAMTS5	0.1	0.8	0.3
MMP1	0.5	0.01	0.6
MMP3	0.1	1	0.1
MMP13	0.8	0.007	0.0001↑
TIMP3	0.005	0.6	0.4
IL1β	0.9	0.6	0.08
COL1A2	0.2	0.0001↑	0.0001↑
COL2A1	0.6	1	0.5
COL10A1	1	0.2	0.1
ACAN	0.002↓	0.002↓	0.0004↓
BGN	0.09	0.02	0.2

Table 7.3: Significance of differences in gene expression in cartilage of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD cartilage, as calculated using mixed effects linear regression, significance level P≤0.004. Arrows show whether gene expression was up- or down-regulated.

Subchondral Bone

In subchondral bone, as compared to samples unaffected by POD (Grade 0), there was a significant increase in expression of *COL2A1* in POD Grade 3 samples (P=0.0001) (Figure 7.5). There was a trend towards an increase in expression of *COL2A1* in POD Grade 1 and POD Grade 2 samples (P=0.006 and

P=0.005 respectively), but these just failed to reach statistical significance. Likewise, there was a trend towards an increase in *COL1A2* expression in POD Grade 1 and POD Grade 3 samples (P=0.006 and P=0.009 respectively).

As compared to Grade 0 POD samples, there was a significant decrease in expression of *BGN* in POD Grade 1 (P=0.004) and POD Grade 2 (P=0.002) samples (Figure 7.6).

Significance of differences in gene expression in cartilage of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD are shown in full in Table 7.4.

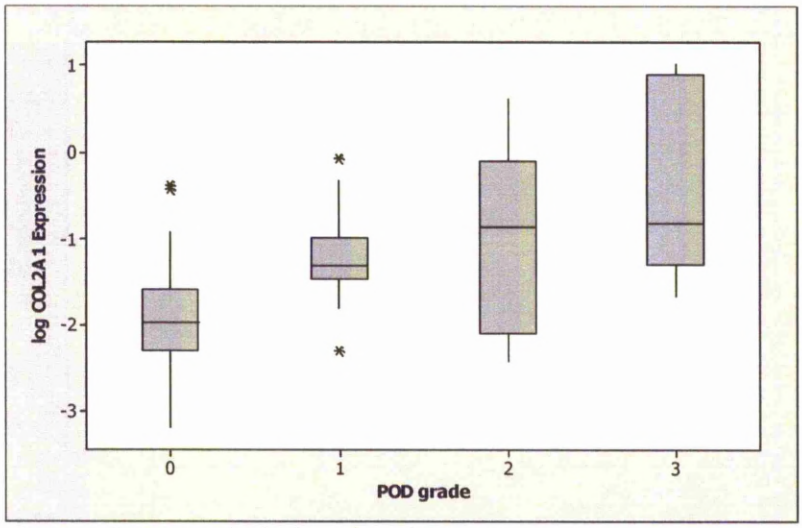

Figure 7.5: Box and whisker plot of COL2A1 expression in subchondral bone affected by each grade of POD. Asterisks show outlying results. Expression of COL2A1 was significantly increased in subchondral bone from Grade 3 POD samples (P=0.0001). There was a trend towards an increase in COL2A1 expression in subchondral bone from Grade 1 POD samples (P=0.006) and Grade 2 POD samples (P=0.005).

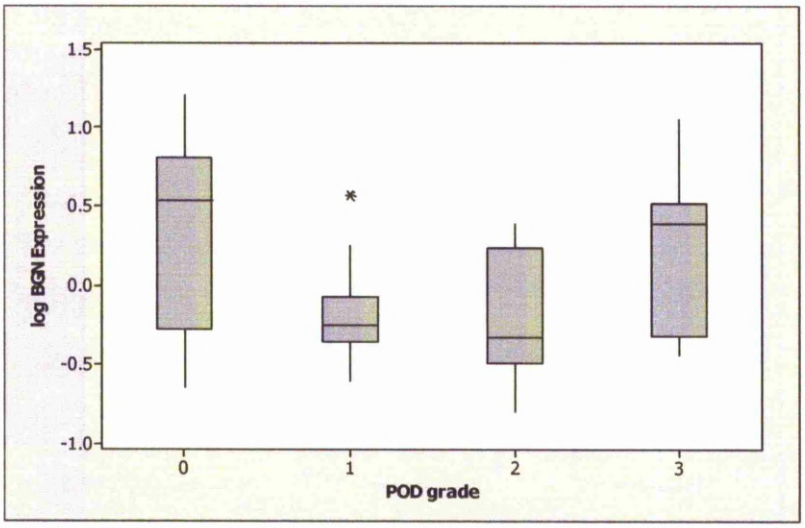

Figure 7.6: Box and whisker plot of BGN expression in subchondral bone affected by each grade of POD. Asterisks show outlying results. Expression of BGN was significantly decreased in subchondral bone from Grade 1 POD samples (P=0.004) and Grade 2 POD samples (P=0.002).

Subchondral Bone			
Gene	POD Grade 1	POD Grade 2	POD Grade 3
ADAMTS4	0.6	0.9	0.5
ADAMTS5	0.7	0.9	0.8
MMP1	0.05	0.08	0.04
MMP3	0.2	0.6	0.04
MMP13	0.3	0.5	0.2
TIMP3	0.02	0.6	0.04
IL1β	0.08	0.2	0.4
COL1A2	0.006	0.8	0.009
COL2A1	0.006	0.005	0.0001↑
COL10A1	0.4	0.8	0.1
ACAN	1	0.4	0.9
BGN	0.004↓	0.002↓	0.5

Table 7.4: Significance of differences in gene expression in subchondral bone of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD subchondral bone, as calculated using mixed effects linear regression, significance level P≤0.004. Arrows show whether gene expression was up- or down- regulated.

Synovial Membrane

No significant differences were found in expression of any genes examined in synovial membrane, as shown in Table 7.5.

Synovial Membrane			
Gene	POD Grade 1	POD Grade 2	POD Grade 3
ADAMTS4	0.3	0.5	0.06
ADAMTS5	0.9	0.03	0.5
MMP1	0.7	0.9	0.2
MMP3	0.4	0.8	0.3
MMP13	0.3	0.08	0.9
TIMP3	0.7	0.4	0.9
IL1β	0.1	0.4	0.2

Table 7.5: Significance of differences in gene expression in synovial membrane of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD synovial membrane , as calculated using mixed effects linear regression, significance level P≤0.007.

7.3.3 Correlation in Expression of Genes between Tissues

In synovial membrane and SCB, a significant correlation was found between expression of *IL1B* (P=0.01, r=0.3) (Figure 7.7). In synovial membrane and cartilage, a significant correlation was found between expression of *MMP1* (P=0.002, r=0.4) and *ADAMTS4* (P<0.001, r=0.5). In subchondral bone and cartilage, significant correlations were found between expression of: *MMP1* (P=0.02, r=0.4); *MMP3* (P<0.001, r=0.5); *ADAMTS4* (P<0.001, r=0.6); *ADAMTS5* (P=0.001, r=0.4); *COL1A2* (P=0.04, r=0.3); *COL2A1* (P<0.001, r=0.5); *COL10A1* (P=0.008, r=0.3) and *BGN* (P<0.001, r=0.8). The correlation between SCB and cartilage biglycan expression is shown in Figure 7.8.

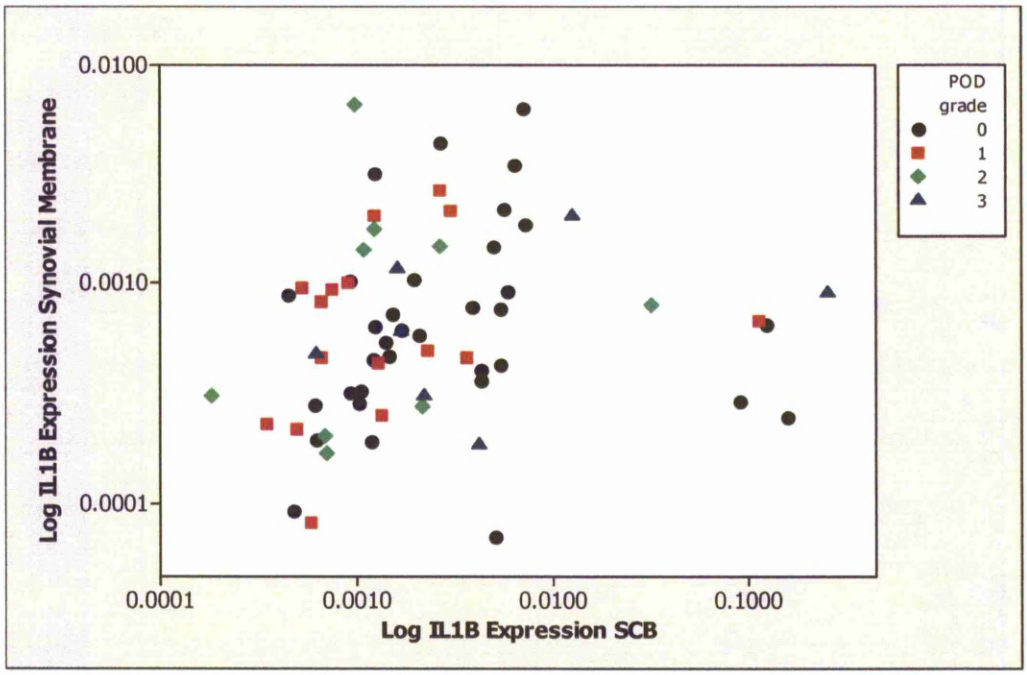

Figure 7.7: Scatterplot showing significant correlation between IL1B expression in synovial membrane and subchondral bone (P=0.01, r=0.3).

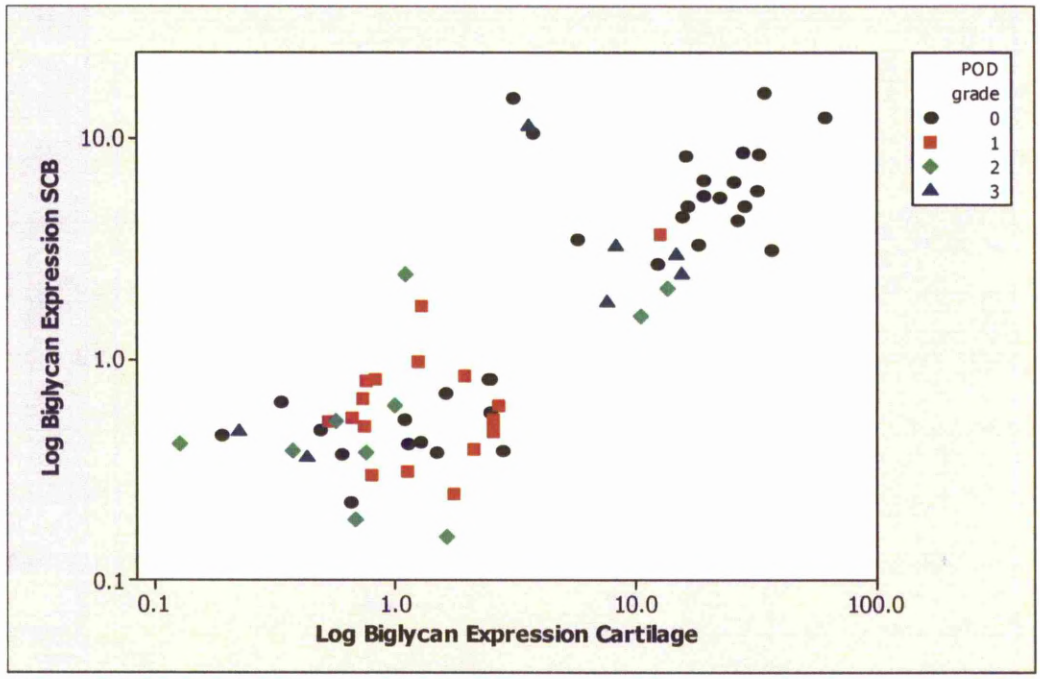

Figure 7.8 Scatterplot showing significant correlation between biglycan expression in cartilage and subchondral bone (P<0.001, r=0.8).

7.4 Discussion

By quantifying and comparing the gene expression of various proteolytic enzymes and structural proteins, it has been shown that there are alterations in gene expression in both the cartilage and SCB of condyles affected by palmar osteochondral disease as compared to those unaffected by pathology. Further, scoring the condyles at post-mortem examination using a well-defined system gave some indication as to the severity and stage of the disease. It is proposed that the Grade 1 POD samples represent the result of early disease where the SCB is bruised and may be mildly fragmented but the HAC is intact, Grade 2 a middle stage of disease where the SCB has failed but there is not yet collapse of the overlying HAC, and Grade 3 POD samples an end-stage or severe disease where both the SCB and HAC have failed. By analysing gene expression data separately for each grade of POD it was possible to consider stages of disease separately and crucially to allow the possibility of identifying biomarkers for the early potentially reversible stages of disease. This data may give some further understanding as to the pathogenesis of this debilitating racehorse condition.

MMP13 expression was significantly increased in the HAC of condyles affected by Grade 3 POD, with a trend toward increased expression in Grade 2 POD samples. MMP13 is often cited as one of the most important mammalian collagenases and is known to be important in the pathophysiology of osteoarthritis (Cawston and Wilson 2006) and it would appear to be involved only in the later and more severe stages of disease in the articular cartilage of horses affected by POD. There are obvious limitations in looking at quantitative gene expression of MMPs in isolation and attempting to draw conclusions as to the levels of active enzymes in the tissue. This is because after gene transcription, the proteins must first be translated and then cleaved before secretion in their active form.

The failure to identify any alteration in expression of various matrix metalloproteinases in the synovial membrane differs to previous findings in synovium from osteoarthritic joints by Davidson et al. (2006). This may be explained by species differences, the small panel of biomarkers examined in

this study as compared to microarray studies and to differences in controls i.e. our POD negative horses had also been in training and therefore may have had underlying joint pathology.

ACAN expression was found to be significantly decreased in all grades (1, 2 and 3) of POD. Aggrecan is the major proteoglycan in cartilage and in arthritic cartilage aggrecan and type II collagen are degraded and then lost, resulting in a thin and mechanically weakened tissue. Aggrecan loss from cartilage is considered to be an early (Pond and Nuki 1973) and reversible (Thomas 1956) event in the pathogenesis in OA and aggrecan may have a protective role in preventing collagen degradation (Pratta *et al.* 2003). It is interesting that in this disease aggrecan synthesis of HAC as measured by gene expression, was found to be decreased at all stages of disease.

Recently much research interest has been generated by the identification of the A Disintegrin and Metalloproteinase with Thrombo Spondin motifs (ADAMTS) gene family. The purification of ADAMTS4 from bovine cartilage (Tortorella *et al.* 1999) was closely followed by the identification of ADAMTS5 (Abbaszade *et al.* 1999). There remains some debate as to the relative importance of each aggrecanase in the pathogenesis of OA, and to some extent this appears to vary with species (Fosang *et al.* 2008; Huang and Wu 2008). I showed that neither ADAMTS4 nor ADAMTS5 expression were altered in either the HAC or SCB in POD and this may be surprising given previous evidence as to the importance of aggrecanase activity in OA. Potentially this may be explained either by the constraints of the technique of looking at relative quantification of gene expression in isolation, with identification of neoepitopes of aggrecan in cartilage and subchondral bone possibly being a more accurate means of identifying ADAMTS activity in the samples.

It was also interesting that although *ADAMTS4* and *ADAMTS5* gene expression were not altered in disease, there was a significant correlation between their expression in SCB and HAC, which may suggest a similar mechanism of regulation in these osteochondral tissues. There was also correlation

in expression of other proteolytic enzymes (*MMP1, MMP3*) and of structural proteins (*COL1A2, COL2A1, COL10A1* and *BGN*) in cartilage and SCB which further demonstrates an apparent synchrony in gene expression between HAC and SCB, not necessarily associated with disease.

There was a significant increase in expression in *COL1A2* expression in POD Grade 2 and 3 cartilage and a trend towards increased expression of *COL1A2* in POD Grade 1 and 3 SCB. This may suggest that the cartilage in late stage disease is attempting to heal by fibrocartilage formation. In the subchondral bone, there is a biphasic response whereby the bone is attempting healing in the early stages, and then again in the late stages of disease.

There was a significant increase in *COL2A1* expression in the SCB of POD Grade 3 samples and a trend towards an increase in *COL2A1* expression in the subchondral bone of Grade 1 and Grade 2 SCB. *COL2A1* expression was not altered in HAC of any grade of POD. This may suggest that in all stages of diseases, but particularly in late stage disease, there is an attempt by the subchondral bone to heal by fibrocartilage and callus formation. These findings correspond with the histological appearance of the disease as described in Chapter 6 (Histology of Palmar Osteochondral Disease).

A reduction in biglycan expression in SCB with POD grades 1 and 2 may indicate a reduction in matrix synthesis in SCB in early stage disease. Biglycan, along with other proteins such as decorin, lumican and fibromodulin, is a small leucine-rich proteoglycan (SLRP) and has been found to interact with collagens (Raspanti *et al.* 2008; Sugars *et al.* 2003; Vynios *et al.* 2001). Biglycan has also been found to be important in the mineralisation of bone with the biglycan knock-out mouse showing an osteoporosis-like phenotype with a decreased growth rate and decreased bone mass compared to the wild type (Xu *et al.* 1998). More recently, biglycan has also been shown to have the ability to bind, but not activate complement (Sjoberg *et al.* 2009) and the authors proposed the role of SLRPs in the regulation of complement activation in diseases involving extracellular matrix, particularly

those characterised by chronic inflammation, such as osteoarthritis. Our finding of reduced biglycan expression in SCB affected with palmar osteochondral disease contradicts the previous findings of Young et al. (2005) who showed that in an ovine meniscectomy model of osteoarthritis there was upregulation of expression of biglycan. This may be due to species difference, or to the differences in the stage of disease, or indeed to differences in pathogenesis of POD as a manifestation of osteoarthritis.

7.5 Conclusion

This data gives further evidence of the importance of subchondral bone disease in the pathophysiology of OA and is of interest since in this naturally occurring model of OA both grossly and histologically the SCB is affected earlier in the course of disease than the overlying hyaline articular cartilage. Differences in gene expression in cartilage and SCB at different stages and severities of disease were identified. The finding of correlation in gene expression of various structural proteins and proteolytic enzymes in SCB and HAC is also of interest as this data suggests synchrony between expression of certain genes in the two closely associated joint structures.

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Measurement of Markers of Osteogenesis and Osteoclastic Activity in the Subchondral Bone of Horses Affected by Palmar Osteochondral Disease

8.1 Introduction

The results of quantification of gene expression of various enzymes involved in matrix proteolysis and of various matrix proteins in the hyaline articular cartilage, subchondral bone and synovial membrane have been presented in Chapter 7 (Association Between Markers of Osteoarthritis in Subchondral Bone, Cartilage and Synovial Membrane in Horses with Palmar Osteochondral Disease). As I had previously found evidence of alterations in the expression of various bone matrix proteins (*COL2A1*, *COL1A2* and *BGN*) in subchondral bone from condyles affected by palmar osteochondral disease (POD), and because POD is considered to be a form of osteoarthritis in which pathology is first manifested in the subchondral bone (SCB), the quantitative mRNA expression of a further panel of genes involved in osteogenesis and osteoclastic activity in samples of SCB was measured in an attempt to investigate further the pathogenesis of this condition.

Like cartilage, bone is composed of cells and their associated extracellular matrix. Bone extracellular matrix comprises organic (35%) and inorganic (65%) components. The organic component includes predominantly type I collagen (90%), non-collagenous proteins, glycosaminoglycans and lipids. The inorganic component consists mainly of crystalline calcium and phosphate present in the form of hydroxyapatite (Martin *et al.* 1988). Non mineralised bone matrix is synthesised by osteoblasts, cells derived from mesenchymal origin (Martin *et al.* 1988). When matrix synthesis is no longer required, the osteoblast loses its synthetic ability and either becomes entombed in its lacuna within the mineralised bone matrix as an osteocyte; transforms into an inactive osteoblast and becomes a bone –lining cell; undergoes apoptosis; or transdifferentiates into cells that deposit chondroid (Franz-Odendaal *et al.* 2006).

There are a number of non-collagenous bone matrix proteins and only a sample of those will be considered further here. Bone γ-carboxyglutamate protein (BGLAP), also known as osteocalcin, is a small protein synthesised by mature osteoblasts which has three residues of the calcium-binding amino acid γ-carboxyglutamic acid (Poser *et al.* 1980). BGLAP is thought to interact directly with hydroxyapetite through its Gla residue (Hauschka *et al.* 1989). Osteomodulin (OMD), also known as osteoadherin, is a small leucine-rich proteoglycan thought to mediate cell attachment via the amino acid sequence Arg-Gly-Asp (RGD) (Sommarin *et al.* 1998; Wendel *et al.* 1998). Secreted phosphoprotein 1 (SPP1), also known as osteopontin and bone sialoprotein I, is a bone matrix protein containing relatively large amounts of sialic acid as well as the RGD cell attachment sequence and is a member of the Small Integrin-Binding Ligands with N-linked Glycosylation (SIBLINGs) family. SPP1 binds with high affinity to hydroxyapatite and it has been suggested that it participates in tissue mineralisation (Oldberg *et al.* 1986). Like SPP1, integrin-binding sialoprotein (IBSP), also known as bone sialoprotein II, is a member of the SIBLINGs family. In contrast to SPP1, IBSP exhibits a more limited pattern of expression and is thought to mark a late stage of osteoblastic differentiation and early tissue mineralisation (Bianco *et al.* 1991).

While excessive bone resorption may be pathological, a balance between bone formation and resorption is necessary for effective bone homeostasis and physiological adaptation of the bone to the stresses applied by load and exercise. As described above, osteoblasts have a primary function in bone formation, whereas bone resorption is effected primarily through the osteoclast. Osteoclasts are derived from mononuclear precursors and are formed by the fusion of cells of the monocyte-macrophage line (Martin *et al.* 1988). Osteoclasts are richly endowed with lysosomal enzymes, especially acid phosphatase 5 tartrate resistant (ACP5), also known as tartrate-resistant acid phosphtase, which is considered to be specific for osteoclasts (Eggert 1980; Minkin 1982) and thus ACP5 is used widely as a marker of osteoclasts. Although less tissue specific in its expression than ACP5, cathepsin K (CTSK) is a cysteine proteinase which has also been shown to be highly expressed in osteoclasts (Bossard *et al.* 1996; Drake *et al.* 1996). The role of the osteoclast in bone

resorption was further defined in the mid to late 1990s when the identification of the receptor activator of nuclear factor-κβ ligand (RANKL)/RANK/osteoprotegerin (OPG) signalling system clarified the means by which the osteoblast was able to regulate osteoclast formation (Anderson *et al.* 1997; Lacey *et al.* 1998; Simonet *et al.* 1997; Yasuda *et al.* 1998a; Yasuda *et al.* 1998b).

The aim of the following experiment was to investigate the pathogenesis of POD further by comparing the expression of various genes involved in osteogenesis and osteoclastic activity in the subchondral bone (SCB) of condyles from Thoroughbred racehorses at various stages of disease. It was hypothesised that there would be an increase in expression of non-collagenous matrix protein genes in early disease (Grade 1 POD) with a reduction in expression of non-collagenous matrix protein genes in later/more severely affected SCB (Grades 2 and 3). It was also hypothesised that there would be an increase in expression of genes involved in osteoclastic activity in SCB affected by POD, particularly more severe grades of POD (Grades 2 and 3), as compared to SCB unaffected by POD (Grade 0).

8.2 Materials and Methods

Tissue collection, RNA extraction, reverse transcription and quantitative real-time PCR were performed as before (Chapter 7). cDNA from SCB derived from those previous samples was used again in this set of experiments. Gene expression of *BGLAP*, *OMD*, *SPP1*, *IBSP*, *RANK*, *RANKL*, *OPG*, *CTSK* and *ACP5* were measured relative to *GAPDH* as a housekeeping gene. Primers used for target and housekeeping genes are as shown in Table 8.1.

Gene	Primer Sequence	Accession Code	Efficiency
GAPDH	F: GCATCGTGGAGGGACTCA R: GCCACATCTTCCCAGAGG	AF157626	-3.32
BGLAP	F: TCAACCCAGACTGTGACGAG R: CAGCTAGGGACGATGAGGAC	XM_001915727	-3.80
OMD	F: CAAATTCATCAACCCCTGAAA R: CTTCATCTGGCTCTTGGTCA	NM_005014	-3.19
SPP	F: CCAGGAAGTTTCGCAGATCTG R: CACAGGTGATGTGAGGTCCTCTT	XM_001496152	-3.36
IBSP	F: TTATCCTCCTCTAAAACGATTTCCA R: CTATCACCATCTCCATTTTCTTCAGA	XM_001496125	-3.25
RANK	F: GCTTCACTGGGACAGAGAACATG R: GCGCTCGGCGAAGTTG	NM_003839	-3.49
RANKL	F: CCGTGGAAGGTTCTTGGTTA R: ATGATGGCGAAAGCAAATGT	XM_001915535	-3.87
OPG	F: AGTGAATCAACTCAGAAATGTGGAAT R: AGGAACAGCAAACCTGAAGAATG	NM_002546	-3.20
CTSK	F: ATGGGATCCAGAAGGGAAAC R: TGGCTGAAGTCACATCTTGG	XM_001490599	-3.3
ACP5	F: ACCCACTGCCTCGTCAAG R: GTCGAGGGGTCCATGAAGT	XM_001490293	-3.33

Table 8.1: Primer sequences used for quantitative real-time PCR

F= Forward primer, R= Reverse primer

Samples were scored grossly for grade of POD at *post mortem* examination as described previously (see Chapters 5 and 7) and grouped according to this classification for further statistical analysis. Data was plotted and tested for normality using the Kolmogorov-Smirnov test (Minitab v.15, Minitab Inc., Pennsylvania, USA) and mixed effects linear regression (SPlus v6.1, TIBCO Software Inc., California, USA) was used to test for significant differences between groups while allowing for clustering within horse. To allow for testing of multiple genes on the same sample, the P value was adjusted using Sidak's formula (Sidak 1967):

$$\alpha_{PT} = 1 - (1-\alpha_{PF})^{1/c}$$

as described in full in Chapter 7.

Gene expression data are presented as previously as a box and whisker chart, with tops and bottoms of boxes demonstrating third and first quartiles respectively. The line within the box indicates the median value. Whiskers extend to the highest and lowest data values.

8.3 Results

As in Chapter 7, samples of SCB from 70 distal metacarpal and metatarsal condyles (35 left fore medial and 35 left hind lateral) were collected at post mortem examination from 35 horses. All horses were Thoroughbred geldings which had been in flat race training. Thirty four condyles (48.6%) had Grade 0 POD, 18 condyles (25.7%) had Grade 1 POD, 11 condyles (15.7%) had Grade 2 POD and 7 condyles (10%) had Grade 3 POD.

Nine genes were measured in each sample of SCB, giving a Sidak corrected significance level of $P \leq 0.006$.

In SCB affected with Grade 2 POD, *BGLAP* expression was found to be significantly reduced (P=0.005) as compared to Grade 0 (POD unaffected) samples (Figure 8.1). There was no statistically significant difference (P>0.006) between grades 1 and 3 POD samples and unaffected samples on *BGLAP* expression or on any grade of POD affected sample and unaffected groups on expression of *OMD*, *SPP1*, *RANK*, *RANKL*, *OPG*, *CTSK* and *ACP5* in subchondral bone.

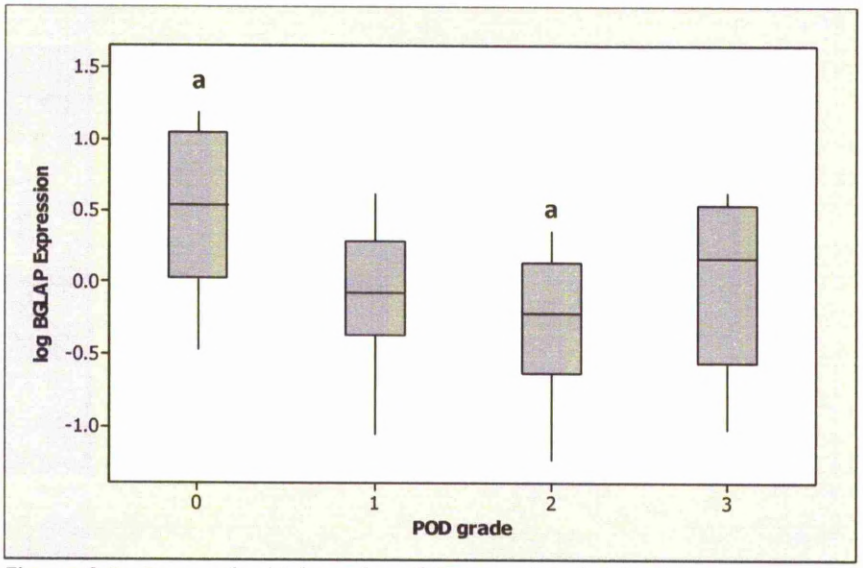

Figure 8.1: *Box and whisker plot of Osteocalcin (BGLAP) expression in subchondral bone affected by each grade of POD. BGLAP expression was significantly reduced in subchondral bone affected by Grade 2 POD as compared to Grade 0 POD (P=0.005), marked by letter (a).*

Gene	POD Grade 1	POD Grade 2	POD Grade 3
BGLAP	P=0.02	P=0.005 ↓	P=0.1
OMD	P=0.3	P=0.1	P=0.3
SPP1	P=0.8	P=0.8	P=0.4
RANK	P=0.5	P=0.02	P=0.3
RANKL	P=0.2	P=0.4	P=0.3
OPG	P=0.2	P=1	P=0.2
CTSK	P=0.2	P=0.5	P=0.1
ACP5	P=0.3	P=0.02	P=0.2

Table 8.2: Significance of differences in gene expression in subchondral bone of Grades 1, 2 and 3 POD for each gene examined as compared to Grade 0 POD subchondral bone. Arrows show whether gene expression was up- or down-regulated.

8.4 Discussion

Alterations in expression of the genes involved in bone matrix synthesis and osteoclastic activity measured in this series of experiments were less extensive than was hypothesised. In the panel of genes examined, each comparing Grades 1, 2 and 3 POD to Grade 0 POD, the only significant finding was that *BGLAP* expression was decreased in subchondral bone from Grade 2 POD samples. *BGLAP* expression was also reduced in Grade 1 SCB POD samples, but this failed to reach statistical significance. The failure to identify extensive alterations in the expression of the other non-collagenous matrix proteins measured (*OMD* and *SPP1*) in SCB at any stage of disease or BGLAP in Grades 1 and 3 POD SCB, may indicate that alterations in bone matrix synthesis are not involved in the pathogenesis of POD. This finding is in contrast to previous studies in osteoarthritis in humans which have shown an elevation in mRNA expression of *BGLAP* and *SPP1* in the subchondral bone of the proximal femur affected by OA (Truong *et al.* 2006).

Clinically POD has been reported to be a difficult condition to treat (Ross 2003) with an apparent slow healing rate of subchondral lesions, if indeed the subchondral bone does heal at all. The lack of alteration in expression of genes involved in non-collagenous matrix synthesis may reflect an inability of the osteoblasts in SCB to respond to insult at the early, mid or late stages of POD. It is

suggested therefore that the lack of increased synthetic activity of the osteoblasts may explain the apparent failure of healing of the subchondral bone in this disease. Another explanation for the lack of alterations in expression of non-collagenous matrix proteins is that alterations in synthesis of bone matrix are not a feature of the pathogenesis of POD. This data may therefore support the hypothesis of previous authors that POD is a structural disease resulting from functional adaptation of the subchondral bone and the development of fatigue microcracking (Muir *et al.* 2008; Norrdin *et al.* 1998; Norrdin and Stover 2006).

Differences in expression of any of the genes involved in osteoclastic activity measured (*RANK, RANKL, OPG, CTSK, ACP5*) were not identified. This may indicate that increased osteoclastic activity is not a feature of the pathogenesis of POD. This may be surprising as although the involvement of the RANKL/RANK/OPG system in the pathogenesis of bone disease in the equine has not been previously reported, the importance of increases in the RANKL: OPG ratio are known to be critical in the pathogenesis of numerous human bone diseases resulting from increased bone resorption including osteoporosis, Paget disease, rheumatoid arthritis and meyeloma (Hofbauer and Schoppet 2004). Likewise, there has also been recent evidence of the importance of CTSK in bone resorption and osteoarthritis with involvement of cathepsins in bone resorption having been indicated in inhibition studies (Delaisse *et al.* 1984; Montenez *et al.* 1994). More specifically, CTSK knock-out mice were found to have an osteopetrotic phenotype (Saftig *et al.* 2000; Saftig *et al.* 1998). In the equine, as in other species, CTSK has been immunolocalised not just to the osteoclast but also to articular chondrocytes (Gray *et al.* 2002), and more recently CTSK has been shown to degrade articular cartilage in naturally occurring equine osteoarthritis (Vinardell *et al.* 2009). *ACP5* expression may have been expected to be altered in POD where subchondral bone lysis is a feature of disease. However, although gene expression of *ACP5* in the equine has not been described, our findings are supported by a previous study comparing osteoarthritic to osteoporotic subchondral

bone in humans where no significant differences in expression of either *ACP5* or *CTSK* were found between groups (Logar *et al.* 2007).

Another possible reason for failing to find statistically significant differences between groups in expression of genes involved in bone matrix synthesis and osteoclastic activity in subchondral bone, is that samples sizes were too small, resulting in a lack of power in the study and therefore a failure to identify significant differences between groups. In addition, by adjusting the significance level using Sidak's method to allow for multiple comparisons, and thereby reducing the type I error, the type II error has been inadvertently increased (Rothman 1990). A type II error occurs where the null hypothesis (the hypothesis that two factors are unrelated and any apparent relation in the data is attributable to chance) is not rejected despite the presence of a true difference between groups.

In this chapter bone matrix synthesis and osteoclastic activity in subchondral bone were examined at gene expression level only, and significant differences were not identified between many of the groups. It may have been useful also to have considered protein levels (e.g. using Western blots) as due to post-translational control there is not necessarily a linear relationship between mRNA and protein levels. The subchondral bone was dismembranated as the first step in mRNA extraction, therefore average gene expression of all the cells in subchondral bone was attained. Since osteoclasts make up only a small percentage of these cells, techniques such as immunohistochemistry or in-situ hybridisation may have been more sensitive in detecting cells with osteoclastic phenotypes.

8.5 Conclusion

It has been shown that the subchondral bone of horses affected with POD at various stages of disease compared to horses not affected with POD show a reduction in *BGLAP* expression in Grade 2 POD SCB. Otherwise there were no alterations in expression of various non-collagenous bone matrix proteins, nor of various genes associated with osteoclastic activity. This would suggest that there is little alteration of the biosynthetic activity of osteoblasts and osteoclasts at any stage of disease. Furthermore, it may be concluded that the changes in the subchondral bone in POD occur as a result of structural alterations of the bone rather than due to metabolic alterations in the cells associated with the bone matrix.

8.6 References

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Concluding Remarks

The thesis had two main themes, the first being the development of an *in vitro* co-culture system for equine osteoblasts and chondrocytes and the second the investigation of various aspects (post mortem findings, histology, and gene expression of chondrocytes, synovial membrane and subchondral bone) of palmar osteochondral disease, a naturally occurring equine disease which affects both bone and cartilage.

In Vitro Co-Culture

Ideally, the pathogenesis of osteoarthritis, or indeed any of any disease, would be investigated in the species of interest. However, in the study of human osteoarthritis normal or mildly affected tissue is rarely available, with most tissue being harvested at joint prosthesis surgery and therefore by definition is severely affected end-stage tissue. While the disease process in distinct species will not necessarily follow exactly the same physiological pathways, very often cross- species comparisons are necessary and *in vitro* animal models are invaluable in OA research because a range of stages of disease and severities of disease can be re-created.

Unlike in the human field, in equine OA research the sourcing of mildly affected and normal tissue is more readily achievable. Various equine models of osteoarthritis have been described previously (Frisbie *et al.* 2002; Gustafson *et al.* 1992; Simmons *et al.* 1999; Todhunter *et al.* 1996) but have disadvantages in terms of expense and morbidity of horses. Further, these models of OA are in general more aggressive than the naturally occurring disease and therefore do not necessarily replicate clinical OA. Various *in vitro* cell culture systems for equine chondrocytes have been described e.g. suspension (Richardson and Dodge 2000), monolayer (David *et al.* 2007) or explant (Busschers *et al.* 2010) cultures and these are attractive as material may be collected from normal horses, either euthanased for other reasons, or obtained from abattoirs. The possibility of passaging

or storing frozen cells for use in suspension cultures means that a large amount of material may be harvested from one animal, thus reducing mortality.

Shortcomings of the Equine Osteoblast and Chondrocyte Co-Culture System

The rate-limiting step in the setting up of the equine osteoblast and chondrocyte co-culture system was found to be the growth of primary cells from bone explants (data not shown). The time for the cells to grow out of the explants was found to be highly variable between donors, taking on average 2-4 weeks. This variability in outgrowth of primary cells had no obvious reason (e.g. age of donor) and this variability unrelated to age of donor was also described by McDuffee et al. (2006).

Increased tissue availability can be achieved by cryopreservation of cells. This technique has been utilised with success in other cell culture systems e.g. chondrocytes (Tomford *et al.* 1984). Various cryopreservative procedures for human articular cartilage chondrocytes have been optimised (van Steensel *et al.* 1994). Cryopreservation of osteoblast-like cells has been described with regards to autogenous bone grafts, where the cells were stored within the bone explants (Reuther *et al.* 2006). However, there is a paucity of data regarding cryopreservation of primary cells grown from bone explants. Because the outgrowth of primary cells from the bone explants is the rate-limiting step as discussed above, during the course of the development of the co-culture system it was attempted to cryopreserve the primary cells at first passage after outgrowth from bone explants using standard freezing medium (DMEM, 10% FCS and 10% dimethyl sulphoxide) and slow freezing techniques (Rendal-Vazquez *et al.* 2001). After such cryopreservation the cells failed to grow when culture in monolayer was attempted (data not shown). Therefore for each co-culture experiment osteoblasts had to be differentiated from primary cells grown from bone explants.

Because the osteoblasts had to be grown from primary explants and this took on average 3-4 weeks, it was not possible to use autologous cartilage explants, because the cartilage explants would have

been non-viable by the time they were to be used in the co-culture system if harvested at death of the donor and at the same time as the subchondral bone explants. It would have been possible to cryopreserve the cartilage explants, however ultrastructural changes have been described in articular cartilage chondrocytes associated with freeze thawing (Tavakol *et al.* 1993) therefore it was considered more appropriate to use fresh cartilage explants. For practical reasons outlined above, it was therefore necessary to utilise allogeneic cartilage explants and osteoblasts in the co-culture system. While it may be proposed that this experimental design may result in donor effects and possible aberrant results with regards to expression of proteolytic enzymes as an effect of immunological response against allogeneic tissue, this is unlikely in an *in vitro* system as there is no humoral or cellular response present. Further, because cartilage is devoid of blood vessels *in vivo* it is regarded as immune privileged tissue (Bolano and Kopta 1991) and chondrocytes have been shown to express factors related to immune privilege including; macrophage migration inhibitory factor, Fas ligand, transforming growth factor β and neuropepetides (Fujihara *et al.* 2010). Therefore the experimental design utilising allogeneic osteoblasts and cartilage explants is unlikely to have changed the experimental results and outcomes.

This thesis has outlined the development and validation of a novel osteoblast and cartilage explant co-culture model. It was proposed that this system may serve as an *in vitro* model of equine osteochondral disease, of particular interest given the emphasis of the later part of the thesis regarding the naturally occurring condition of palmar osteochondral disease (POD). Although the system generated interesting data, as an *in vitro* system the co-culture could never absolutely recreate the natural disease process *in vivo*. However, *in vitro* models have advantages over *in vivo* or *ex vivo* studies with regards to minimising morbidity of donor animals, which is of practical benefit with regards to both animal welfare and economics.

Potential for Further Work Utilising the Osteoblast and Chondrocyte Co-Culture System

It is believed that this model may be utilised in the further investigation of osteochondral disease and in the validation of drugs used in treatment of equine joint disease. It is also possible to extend the model to direct co-culture. Indirect co-culture was used here as this is thought to correspond most closely to the situation in vivo. It was shown that soluble factors produced by osteoblasts into the culture medium altered chondrocyte phenotype and resulted in cartilage matrix breakdown effected via the live chondrocyte, most likely via local chondrocyte production of proteolytic enzymes. Another potentially interesting future study would be the comparison of direct to indirect co-culture, particularly with regards to the magnitude of matrix degradation measured by GAG release from the system. Further work is also required to more accurately identify the soluble factors produced by the osteoblasts which drive the altered phenotype.

In this system I utilised IL1β to pre-treat the osteoblasts, thus driving them towards an osteoarthritic phenotype. While IL1β is an important cytokine in the pathogenesis of osteoarthritis, by no means does it act in isolation, and *in vivo* it is likely that a wide variety of cytokines are involved in the disease process. Therefore, use of IL1β alone was potentially an overly simplistic model. Other cytokines which have been shown to be implicated in osteoarthritis include tumour necrosis factor α, IL6, members of the IL6 protein superfamily, IL7, IL17 and IL18 (Malemud 2010). These cytokines, either alone or in combination, could be utilised to drive an osteoarthritic phenotype in the osteoblasts in future experiments.

The co-culture system could be utilised to validate and determine the *in vitro* modes of action of drugs used therapeutically in osteoarthritis, e.g. non steroidal anti-inflammatory drugs, corticosteroids, autologous serum and hyaluronic acid, as well as various nutraceuticals e.g. glycosaminoglycan and chondroitin sulphate. While the use of these agents is well-established, and their mechanisms of action on chondrocytes and cartilage matrix have been defined, few studies

have shown their concurrent effect on osteoblasts and chondrocytes. If osteoarthritis is to be considered as a form of "organ failure" affecting not only the articular cartilage, but also the subchondral bone and synovial membrane, then this data is likely to be extremely useful and clinically relevant.

The technique of metabolomics was used to identify changes in conditioned culture media from the co-cultures and yielded some interesting results. It would have been possible to extend this experiment to identify changes in cartilage alone either treated or not with IL1β, and not just considered the co-culture studies as this work has not to our knowledge been performed previously.

Potential for Further Work Involving the Metabolomic Analysis of Biological Fluids in Osteoarthritis

As stated above, metabolomic analysis was utilised in analysis of conditioned culture media from *in vitro* experiments. Metabolomics however has the scope not only to be used *in vitro*, but also in *in vivo* through analysis of biological fluids. Previously a urinary metabolite profile has been identified in the guinea pig spontaneous OA model (Lamers *et al.* 2005). Similarly, metabolic profiling of synovial fluid in osteoarthritis has been described in canine experimental models of osteoarthritis (Damyanovich *et al.* 1999a; Damyanovich *et al.* 1999b) and in a guinea pig experimental model of osteoarthritis (Borel *et al.* 2009). There is comparatively less data on naturally occurring osteoarthritis, although there is one recently published paper looking at the lipidomics of plasma from human osteoarthritis patients (Castro-Perez *et al.* 2010).

In a future study, it would be interesting to analyse further samples, either of plasma or of synovial fluid, from naturally occurring equine osteoarthritis/joint disease. As detailed in later chapters of the PhD, samples from the palmar condyle of the distal metacarpus and metatarsus were obtained at post mortem examination from a population of Thoroughbred racehorses. Samples of synovial fluid and blood were also obtained at the time of euthanasia in these horses. It will be interesting in

future studies to evaluate the metabolic profile of these samples and to assess whether from these results it is possible to conclude whether horses are suffering from osteoarthritis. This would provide a useful *pre-mortem* test, particularly in those conditions, such as palmar osteochondral disease, which may appear normal on radiographic and arthroscopic examination, and therefore may pose a diagnostic problem for the clinician.

It was found that in the samples of conditioned culture medium from the *in vitro* co-cultures, there were very few peaks after retention times of 15 minutes, indicating that no lipophilic compounds in the media. In comparison, Castro-Perez et al. (2010) identified changes in the lipid profiles of plasma from osteoarthritic patients, and suggested that there was an altered lipid metabolism associated with osteoarthritis and the release of arachidonic acid from phospholipids. The differences between our results are likely to reflect differences in fluids analysed and also differences between naturally occurring and *in vitro* cell culture models of OA. It will be interesting to evaluate the lipophilic compounds in the plasma and synovial fluid samples from the horses affected by naturally occurring joint disease in future experiments extending on from this thesis.

Palmar Osteochondral Disease

The *post-mortem* evaluation of distal metacarpal and metatarsal condyles from a population of Thoroughbreds which had been in race training showed that the condition of palmar osteochondral disease (POD) was more common than possibly first thought. Further epidemiological studies are required to identify the risk factors for this condition and therefore to aid in its prevention. However, it is unclear whether the mild lesions are clinically significant or performance limiting in any way. If *post-mortem*, histology and training and performance data were considered from each horse, it may be possible to assess whether the severity of lesion has an effect on limitation of performance. Histology allows more objective and quantitative analysis of the osteochondral lesions, particularly given our findings that supported the usefulness of both the Mankin and

OOCHAS scoring systems. While the information regarding performance was available for the horses examined grossly at *post mortem* and histologically here, the numbers were too small to draw any statistically significant conclusions. Therefore, a larger study is required, although it is suggested that analysis of this data will be complicated by a plethora of other confounding factors which may result in poor performance in the Thoroughbred racehorse.

It was also not possible to determine whether the lesions identified at *post mortem* were progressive, from the mild to the severely affected forms of disease although this was assumed to be the case. The only way of determining this would be to perform a longitudinal study on a cohort of Thoroughbreds in training, and because it is readily apparent that *post-mortem* evaluation is useless in such a study design, this would require other means of identifying the disease. At present, the preferred means of diagnosing palmar osteochondral disease would appear to be magnetic resonance imaging (MRI) given that the condition is often not evident on radiography. While a longitudinal MRI study would be possible, it may be prohibitively expensive. Further, there are ethical issues to consider regarding whether it would be appropriate to identify a subchondral lesion on MRI (i.e. a Grade 1 or Grade 2 POD lesion) and subsequently allow that horse to continue in training without treatment as would be required to monitor the progression of disease.

Gene Expression

In all qRT-PCR analysis (both for POD samples and samples obtained from the *in vitro* co-culture systems) GAPDH was chosen as the housekeeping gene based on the majority of previous studies on chondrocyte gene expression. Gene expression measurements may vary as a result of number/type of cells in the tissue, the method and efficiency of mRNA extraction, mRNA handling techniques, mRNA integrity, method of reverse transcription and analytical detection chemistry method (Bustin 2002; Bustin and Nolan 2004; Lekanne Deprez *et al.* 2002). A means of addressing these potential variations is to compare the expression of genes of interest to an internal standard or "house-

keeping" gene. Ideally this house-keeping gene would be constitutively expressed, such that the RNA products of such genes would be at constant levels at all times, irrespective of growth conditions (Avison 2007). However, although previously it was thought that genes such as GAPDH and β-actin fulfilled these criteria, there is mounting evidence that in reality there is no single perfect control for any gene expression study as even housekeeping genes are expressed at different levels under different conditions. Further, the RNA products of housekeeping genes are generally abundant, meaning that differential amounts of degradation between RNA samples may have a smaller impact on housekeeping gene mRNA levels than on the target mRNA (Avison 2007). More recently, authors have described the use of statistical algorithms for the optimisation of reference gene selection including geNorm (Vandesompele *et al.* 2002), Global Pattern Recognition (Akilesh *et al.* 2003), Bestkeeper (Pfaffl *et al.* 2004), equivalence testing (Haller *et al.* 2004) and NormFinder (Andersen *et al.* 2004). Use of these methods would potentially have allowed increased specificity and sensitivity of the technique; however they do also add considerably increased complexity and expense to experiments and their use may be precluded by practical constraints in using a 96 well plate system.

Gene Expression in Palmar Osteochondral Disease

The results of gene expression in subchondral bone (SCB), hyaline articular cartilage and synovial membrane from the metacarpo- and metatarsophalangeal joints of Thoroughbred racehorses were shown and discussed in the thesis. Correlations in gene expression in cartilage and SCB were shown, thus suggesting synchrony between expression of certain genes. In further experiments examining subchondral bone of horses affected by POD at various stages of disease as compared to the SCB of horses that had been in training not affected by POD, there was little evidence of changes in expression in various non-collagenous bone matrix proteins, nor of various genes associated with osteoclastic activity. This was concluded to potentially reflect an unaltered biosynthetic activity of osteoblasts and osteoclasts in disease and it was suggested that the pathogenesis of POD is more

likely to reflect structural alterations of the bone as opposed to metabolic alterations in the cells associated with the bone matrix. It is believed that these structural alterations may be further characterised by ongoing scanning electron microscopy studies.

Despite the negative results, it is important to consider bone as a dynamic and living tissue and to consider the responses of its cell population. Although it is widely recognised that bone has the ability to adapt to physiological stresses, and this has been particularly well-described structurally in the equine metacarpophalangeal joint (Easton and Kawcak 2007; Firth *et al.* 2009; Muir *et al.* 2008), there is comparatively less data on the biosynthetic activity of the osteocytes, osteoblasts and osteoclasts. This may in part be due to the difficulties in processing bone as compared to other, more pliable tissues. However, it was found that it was possible to extract mRNA from even very dense equine subchondral bone using the techniques as outlined in the body of the thesis. Although I failed to show changes in expression of non-collagenous proteins, other synthetic properties of the cells in bone may well be important in subchondral bone injury and worthy of future investigation e.g. cell signalling mechanisms in response to exercise and/or subchondral bone injury.

Conclusion

In the introduction of this thesis, various hypotheses were proposed. These are repeated with an outcome as to whether proven or disproven:

1) osteoblasts could be isolated from equine SCB, undergo reliable proliferation and differentiation and respond to IL1β such that the osteoblasts showed an osteoarthritic phenotype *in vitro* (PROVEN)
2) co-culture of equine osteoblasts pre-treated with IL1β with cartilage explants from joints unaffected by OA would result in an altered chondrocyte phenotype and evidence of cartilage matrix degradation (PROVEN)

3) there would be detectable differences in the metabolic profiles of conditioned culture media from various equine osteoblast and equine osteoblast and chondrocyte co-culture models, and that useful biomarkers could be identified after analysis of the spectra (PROVEN)
4) POD would have a high prevalence at *post mortem* examination of a population of intensively raced Thoroughbreds with a predilection sites for these pathologies. Further, that there would be an association between POD and other pathologies affecting the distal condyles of MC/MTIII and a correlation between prevalence of POD and pathologies of the dorsodistal aspect of MC/MTIII, suggestive of MCP/MTP joint hyperextension (PROVEN)
5) POD would have a characteristic and well-defined histological appearance (PROVEN) and that the OOCHAS scoring system would be more reliable in analysis of cartilage pathology in samples of condyles affected by POD (DISPROVEN)
6) there would be increases in gene expression of matrix proteinases in SCB, hyaline articular cartilage (HAC) and synovial membrane affected by POD and alterations in expression of genes involved in matrix synthesis in SCB and HAC (PROVEN IN PART)
7) there would be correlations between expression of genes in the various articular tissues (SCB, HAC and synovial membrane) (PROVEN)
8) there would be an increase in expression of non-collagenous matrix protein genes in early disease (Grade 1 POD) with a reduction in expression of non-collagenous matrix protein genes in later/more severely affected SCB (Grades 2 and 3) (DISPROVEN)
9) there would be an increase in expression of genes involved in osteoclastic activity in SCB affected by POD, particularly more severe grades of POD (Grades 2 and 3), as compared to SCB unaffected by POD (Grade 0) (DISPROVEN)

Therefore it is hoped that the work contained in this thesis will go somewhere to improving knowledge of the role of subchondral bone damage in joint disease and to increasing understanding of a debilitating racehorse condition. Perhaps as with any body of scientific work, all questions

cannot yet be met with answers, and in digging deeper it seems that yet more questions have been unearthed.

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